Volume 24 July 1960 No. 5.

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

ANALYTICAL CHEMISTRY ORGANIC CHEMISTRY BIOLOGICAL CHEMISTRY
FOOD & NUTRITION MICROBIOLOGY & FERMENTATION INDUSTRY
SOIL & FERTILIZER

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Published by the Agricultural Chemical Society of Japan
c/o Faculty of Agriculture, University of Tokyo

The Bulletin of the Agricultural Chemical Society of Japan is published bimonthly to provide original papers on agricultural chemistry.

Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $6.00 for all oversea subscribers including postage fee. Please address order to the agencies.

The Agricultural Chemical Society of Japan is also publishing The Journal of the Agricultural Chemical Society of Japan (Nippon Nôgei-Kagaku Kaishi), printed in Japanese.

AGENCIES

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P. O. Box 605, Tokyo Central
Tokyo, Japan.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 435~443, 1960]

On the Mechanism of Radiation Enhancement of Lethal Effect of Sodium Chloride on Microorganisms

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Received November 18, 1959

In connection with the preceding paper concerning the enhancement of radiation lethal effect by the NaCl treatment, the experimental evidences and the further discussion for the possible mechanism of the enhancement by the post-NaCl treatment are presented.

In order to elucidate the radiation damages of cell constituents and its enhancement by NaCl, the infrared analysis and isotopic experiments were carried out with cells of *S. cerevisiae*, *Z. soya* and *E. coli*.

Consequently, it has been concluded that the possible mechanism may involve the processes of increase in cell permeability by irradiation and of the denaturation of the critical cell constituents by increasing penetration of NaCl and thus the sensitivity of irradiated cells to NaCl may increase. The NaCl sensitive sites are considered not to be restricted to the biological targets for radiation inactivation by the supports of the survival curve analysis based on the target theory and of the isotopic experiment.

INTRODUCTION

In the preceding paper[1], the enhancement of the total lethal effect of radiations on microorganisms by the NaCl action during and after irradiation was reported. It has been suggested that some important damages responsible for the radiation enhancement must occur in irradiated cells, because there is a significant difference of tolerance to NaCl between irradiated cells.

And it was pointed out that this phenomenon is independent of the shape of the survival curve, i.e. whether it is exponential or sigmoidal. In view of the target theory, the fact that such enhancement of total lethal effect was observed even in the case of one-hit type microorganisms suggests that radiation damage in living cells responsible for the above phenomenon may be not restricted to alternative targets because damaged targets cannot be contained in survivors of this type microorganism[2].

Thus, it can be assumed that the non-selective radiation damages within cells associate with enhancement by NaCl, then, may involve the process in which radiation chemical and physicochemical damages of cell constituents occur, and these damages may intensify the toxic action of the salt in some ways.

This paper presents the experimental evidences and the further discussion for the above view concerning the possible mechanism of enhancement of radiation lethal effect by the post-NaCl treatment.

MATERIALS AND METHODS

Microoganisms and microbiological procedure *Saccharomyces cerevisiae* Rasse II, *Saccharomyces cerevisiae* H-336 (diploid), *Zygosaccharomyces soya* ZS-5 and *Escherichia coli* K12 supplied by the Institute of Applied Microbiology of the University of Tokyo were used in this study. The microbiological procedure was performed according to the method described in the preceding

1) Y. Okazawa, M. Namiki, S. Yamashita and A. Matsuyama, This Bulletin, **24**, 235 (1960).

2) R.B. Uretz, *Radiation Research*, **2**, 240 (1955).

paper[1]).

Gamma irradiation The ^{60}Co gamma source in the Institute of Physical and Chemical Research was used. The method of irradiation was similar to the previously described one.

Preparation of acetone dried powder Microorganisms subcultured on the nutrient agar media were harvested with a glass rod and M/15 phosphate buffer. Cells were sedimented and washed by centrifugation. Then the cell suspension was divided into four groups: (1) Untreated control (2) irradiated only (*Z. soya*, 8×10^4r corresponding to 10% survival dose, *E. coli* 3.7×10^4r and 11.3×10^4r corresponding to 1 and 0.0001% survival doses, respectively) (3) treated with NaCl (*Z. soya*, 13% 4°C 18 hours, *E. coli*, 6% 4°C 4 hours) (4) irradiated and then treated with NaCl (each treatment was the same as (2) or (3). After these treatment, cells of each group were washed once with distilled water, and the sedimented cells were added with cold acetone. After 20 minutes treatment with acetone, cells were separated by filtration using Buchner funnel and washed twice by small amounts of cold ether. Then the cells were dried in a vacuum desiccator.

Infrared analysis About 1.5 mg of the acetone dried powder of yeast cells (*Z. soya*) were uniformly smeared with the small amounts of the ordinary distilled water in the area of 5×10 mm on the AgCl plate. After drying of the film the spectra of a series of four preparations were measured at about 40% relative moisture by means of the infrared spectrophotometer, Koken DS 301 (double beam, triple path type). Then each film was deuterated with heavy water moisture (45%, for about 5 hours; purity of heavy water used, 99.8%). The deuterated films were dried by H_2SO_4 after determination of spectra. Finally the film was rehydrogenated by the back exchange reaction with ordinary water moisture (40% for about 18 hours). After the equal periods (17 hours) of back-exchange reaction the spectra of rehydrogenated films were determined.

The measurement of infrared dichroism in the oriented film was also carried out by means of the above spectrophotometer. In order to obtain the uniaxial orientation in the film avoiding any denaturation, the film prepared from acetone dried bacterial powder was combed repeatedly and carefully to the same direction before drying. The spectra of these films prepared from a series of treated and control samples were measured using perpendicularly polarized infrared radiations obtained by the help of the AgCl polarizer. For calculating the dichroic ratio, the apparent optical densities of three bands at 1540, 1650 and 1870 cm^{-1} were determined with parallel and perpendicular radiations respectively and intensities of two bands at 1540 and 1650 cm^{-1} were corrected by discounting the intensity of absorption at 1870 cm^{-1} as the back ground. The dichroic ratio $D_{///}/D_{\perp}$, defined as the ratio of two optical densities which were determined by polarized radiations with electric vector respectively parallel and perpendicular to the orientation direction, was calculated at 1540 and 1650 cm^{-1}. The deuteration and back-exchange of the films were carried out similarly to the method as mentioned above.

Penetration of ^{24}Na and ^{36}Cl into yeast cells Two strains of yeast (*S. cerevisiae* Rasse II and *Z. soya* ZS-5) were cultured at 30°C in a medium, consisting of peptone 3%, dried yeast extract 1% and dextrose 5%. After 24 hours culture, cells were collected by centrifugation, followed by washing three times with the cold phosphate buffer (M/15, pH 6.4), and resuspended in the same buffer. About 30 mg of dried cells were contained in one ml of these suspensions. On the other hand, 2 and 13% solutions of NaCl singly labelled with the definite activities of ^{24}Na or ^{36}Cl were prepared.

Gamma irradiation of cell suspensions were carried out under cooling by ice-water, and immediately after irradiation 1 ml of them and the equal volume of ^{24}Na or ^{36}Cl labelled NaCl solutions were quickly mixed. Then mixed suspensions were allowed to stand at 3~4°C for 60 minutes in ice-water bath. And then, they were centrifuged, and supernatants were discarded using a capillary connected with an aspirator. Sedimented cells were quickly washed twice with 5 ml of cold distilled water by the same way. All of washed and sedimented cells were carefully transferred into standard steel dishes for radioactivity counting, using about 1.5 ml of distilled water, evaporated to dryness under infrared radiation for measuring the radioactivity of ^{24}Na or ^{36}Cl penetrated into cells.

The end-window type Geiger-Müller Counter of Model 100 made by the Institute of Physical and Chemical Research was used for measurement of radioactivity.

Release of ^{32}P from yeast cells Beforehand, *S. cerevisiae* H-336 (diploid) had been cultured at 30°C for 40 hours in the medium as mentioned above, in which about 10 μc of ^{32}P per 100 ml were added to label the yeast cells uniformly with ^{32}P. Then labelled cells were collected, washed four times with the M/15 cold phosphate buffer (pH 6.4) by centrifugation, and resuspended in the same buffer. After the irradiation had been carried out in an ice-water bath with doses of 6×10^4 and 12×10^4r, 5 ml of un-

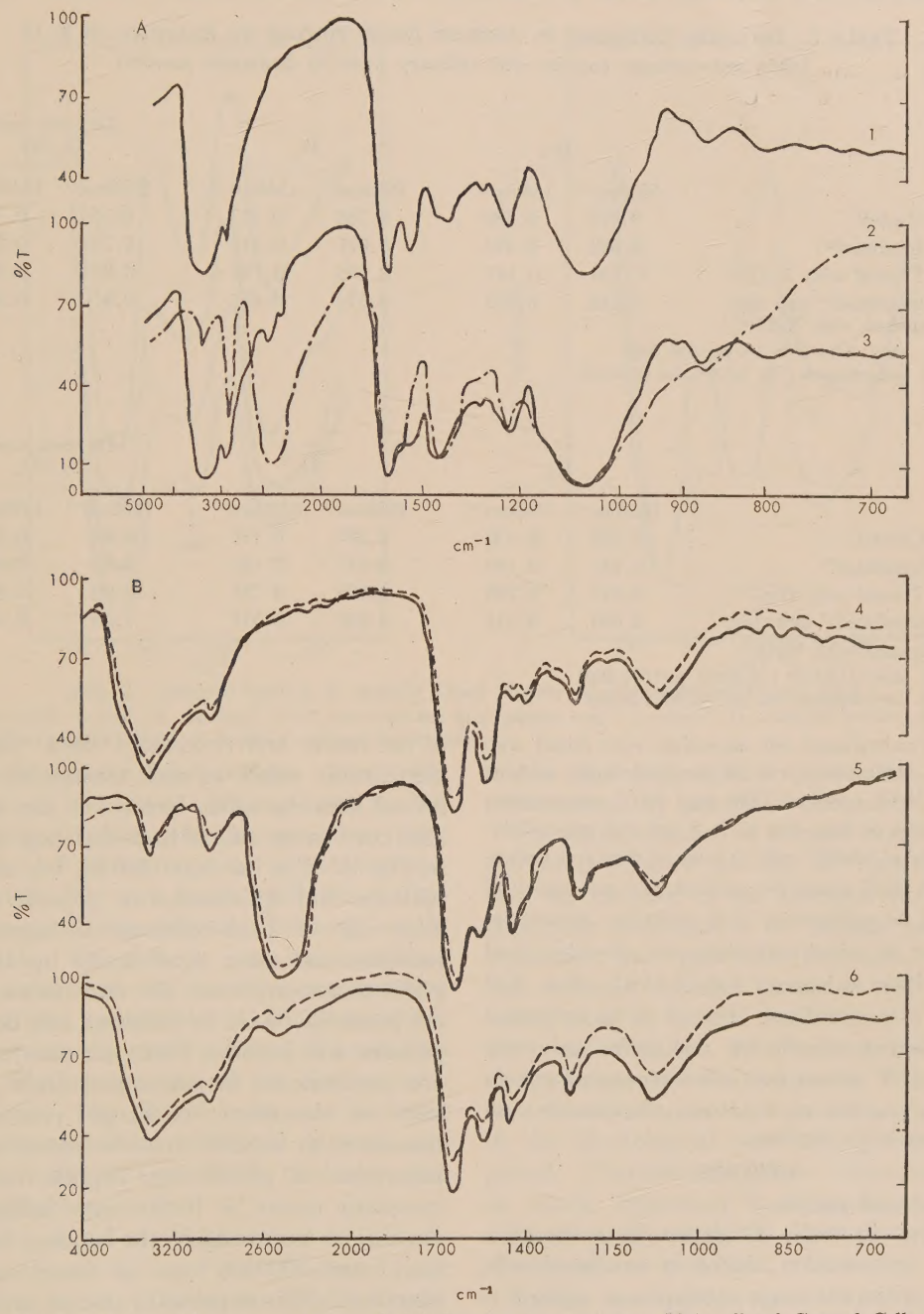

FIG. 1. Infrared Spectra of Acetone Dried Powder Prepared from Unirradiated Control Cells of *Zygosacch. soya* ZS-5 and *Escherichia coli* K12.

A (unpolarized): *Z. soya* 1, native; 2, deuterated; 3, rehydrogenated.
B (polarized): *E. coli* 4, native; 5, deuterated; 6, rehydrogenated.
Broken lines and real lines in B show the spectra measured by the radiations which have the electric vector parallel and perpendicular to direction of orientation, respectively.

TABLE I. INFRARED DICHROISM IN ACETONE DRIED POWDER OF *Escherichia coli* K 12
(after back-exchange reaction with ordinary water in deuterated powder)

Exp. 1

No.		$D_{//}$		$D_{\perp}$		Dichroic ratio $D_{//}/D_{\perp}$	
		1650cm^{-1}	1540cm^{-1}	1650cm^{-1}	1540cm^{-1}	1650cm^{-1}	1540cm^{-1}
1.	Control	0.570	0.329	0.750	0.417	0.76	0.79
2.	Irradiated[a)]	0.682	0.434	0.871	0.512	0.78	0.85
3.	Treated with NaCl[b)]	0.296	0.163	0.376	0.198	0.80	0.82
4.	Irradiated[a)] and then treated with NaCl[b)]	0.518	0.257	0.570	0.261	0.91	0.98

a) dose; 3.7×10^4 r (1% survival dose)
b) concentration; 6% (at 4°C for 12 hours)

Exp. 2

No.		$D_{//}$		$D_{\perp}$		Dichroic ratio $D_{//}/D_{\perp}$	
		1650cm^{-1}	1540cm^{-1}	1650cm^{-1}	1540cm^{-1}	1650cm^{-1}	1540cm^{-1}
5.	Control	0.339	0.150	0.387	0.172	0.88	0.87
6.	Irradiated[c)]	0.343	0.149	0.347	0.158	0.99	0.94
7.	Treated with NaCl[c)]	0.453	0.206	0.506	0.234	0.90	0.88
8.	Irradiated[c)] and then treated with NaCl[d)]	0.699	0.314	0.699	0.317	1.00	0.99

c) dose; 11.3×10^4 r (0.0001% survival dose)
d) concentration; 6% (at 4°C for 4 hours)

irradiated or irradiated cell suspensions were mixed with the equal volume of the M/15 phosphate buffer with or without NaCl addition. The final NaCl concentration of each tube of them was 0, 1, 3 and 5%, respectively. ^{32}P activity of labelled cells in 1 ml of these cell suspensions had 5056 counts per minutes, and supernatant of them had the radioactivity of 55 counts per minutes per ml. After the mixed suspensions were allowed to stand for 40 minutes in ice-water bath (3~4°C), about 3 ml portions were centrifuged. One ml of the supernatant was carefully transferred into steel dishes, and radioactivity of ^{32}P released from cells was measured with the same way described as the above. Experiments were carried out in the duplicate.

RESULTS

The infrared analysis

At first, in order to demonstrate the cytoplasmic denaturation caused in irradiated cells, the deuterium exchange method was applied to the infrared analysis of acetone dried powder. The acetone dried powder prepared from yeast cells (*Z. soya*) indicated no influence of irradiation and/or post-NaCl treatment in the spectra of the native and deuterated films. However, the distinct effect of such treatments was observed with the same band, i.e. the band at 1540 cm^{-1} in the rate of back-exchange as shown in Fig. 2. The band at 1540 cm^{-1} is associated with the NH deformation in peptidic linkage. The rate of back-exchange is increased by radiation and more significantly by the post-NaCl treatment, since the fast back-exchange are primarily found in unfolded and denatured proteins and peptides, whilst the slow exchange are predominant in native molecules[3)]. These data in deuterium exchange reactions are considered to indicate that the same type denaturation of protein and peptide fractions of cytoplasm occurs by irradiation. Influences of the various treatments on the bands at 1650 cm^{-1} ($\nu_{C=O}$) and 3300 cm^{-1} (ν_{N-H}) were not clearly observed. This is probably due to overlapping the other unchanged bands. Secondly, infrared dichroism was measured with the preparations

3) K. Linderstrom-Lang, Symposium on Protein Structure (A. Neuberger, ed.) pp. 23 (9158).

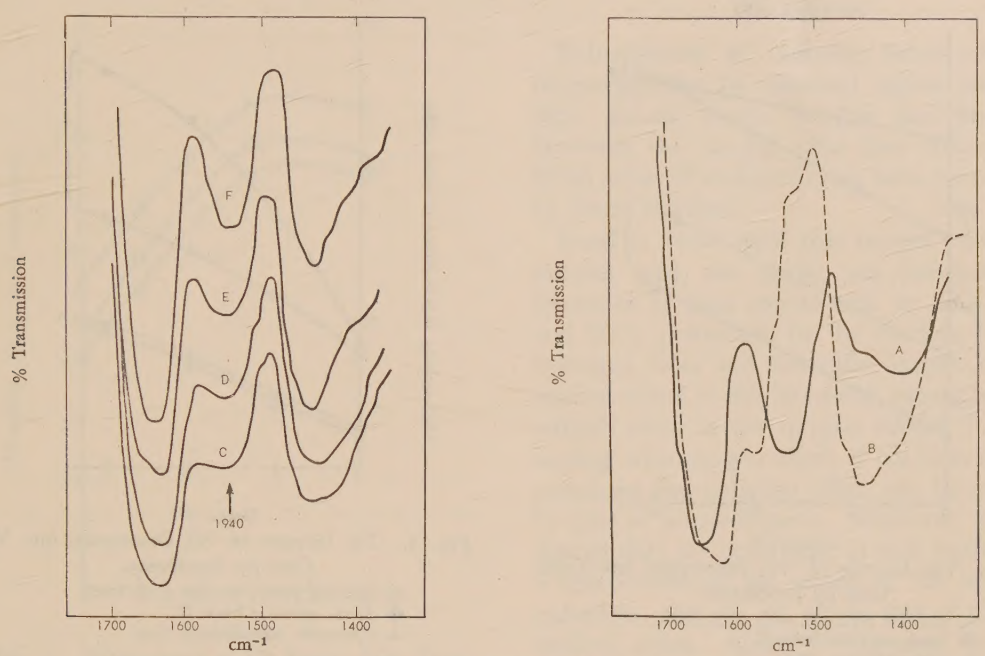

FIG. 2. Infrared Spectra of Acetone Dried Powder of *Zygosaccharomyces soya* ZS-5

A, native (control); B, deuterated; C~F, rehydrogenated with ordinary water; C, control; D, irradiated (8×10^4r); E, treated with 13% NaCl; F, irradiated and treated with NaCl.

of bacterial acetone dried powder. The effect of treatment is observed with two bands at 1540 and 1650 cm^{-1} which may be associated with the NH and CO groups in peptide linkage, respectively. The two bands exhibited the apparent perpendicular dichroism, although not so remarkable. The dichroic ratio was calculated at these two bands with two doses corresponding 1% (3.7×10^4r) and 0.0001% (11.3×10^4r) survival doses, respectively, similar effects of irradiation and or NaCl treatment on the dichroic ratio were found. The dichroic ratio was increased by irradiation and its additional increase was observed with the post-NaCl treatment, the ratio approaching to 1.

In the case of the higher dose irradiation, these two bands decreased the dichroism even without the post-NaCl treatment. Such influence on the mode of the dichroism of the 1540 and 1650 cm^{-1} bands which may associate with the peptide linkage reveals the denaturation, i.e. the disturbance of crystalline configuration of protein or peptide fractions by irradiation and its enhancement by post-NaCl treatment[4].

The results obtained in infrared analysis which are the increase in the rate of back-exchange and the decrease or the disappearance of perpendicular dichroism at the peptide bands by irradiation and post-NaCl treatment, indicate that radiation damages associated with the salt action occur in the cell constituents, at least in the protein fractions. Irradiation and post-NaCl treatment employed in preparing the samples for infrared analysis were similar to those used in the physiological experiment previously reported. Therefore, the above observation has an actual importance in the study on possible mechanism of synergistic interaction between irradiation and post-NaCl treatment.

Further investigation concerning the effect of fractionation of the acetone powder on the dichroic ratio is now under way.

Increased penetration of ^{24}Na and ^{36}Cl caused by

4) G.B.B.M. Sutherland, *Rev. of Modern Phys.*, **31**, 118 (1959).

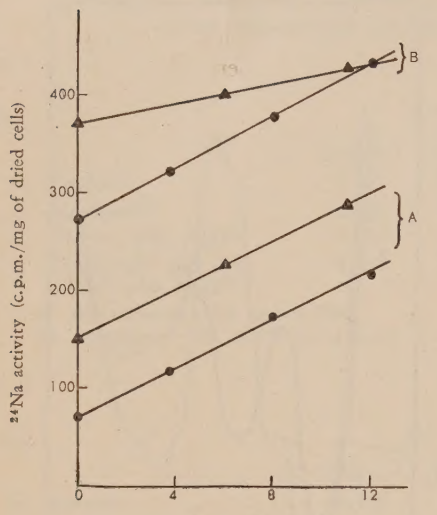

FIG. 3. The Increase of ^{24}Na Penetration into Yeast Cells by Irradiation.

A, with 1% NaCl; B, with 6.5% NaCl;
● *Sacch. cerevisiae* Rasse II
▲ *Zygosacch. soya* ZS-5

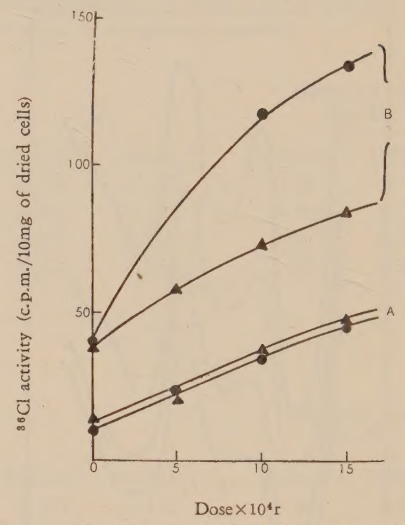

FIG. 4. The Increase of ^{36}Cl Penetration into Yeast Cells by Irradiation.

A, with 1% NaCl; B, with 6.5% NaCl
● *Sacch. cerevisiae* Rasse II
▲ *Zygosacch. soya* ZS-5

irradiation

Penetrations of ^{24}Na and ^{36}Cl into irradiated cells of *S. cerevisiae* and *Z. soya* from saline phosphate buffer containing NaCl singly labelled with ^{24}Na or ^{36}Cl in the concentrations of 6.5 per cent were increased with increasing doses as shown in Figs. 3 and 4. The penetration of ^{24}Na as increased in proportion to increasing doses with the above two strains and the both concentrations of NaCl. As compared with this linear increase of ^{24}Na penetration, somewhat different relationships were obtained with ^{36}Cl penetration. Increase with increasing doses of ^{36}Cl penetration was not linear but of saturation type, especially with the higher NaCl concentration (6.5%).

With the higher NaCl concentrations (6.5%), the increasing ratios of Na and Cl penetrations into cells of the two strains with increasing doses were not equal, although with the lower NaCl concentration (1.0%) they were almost the same. The increasing ratio of *S. cerevisiae* was larger than that of *Z. soya*, NaCl resistant yeast, with the higher NaCl concentration (6.5%). However, the further investigation is needed to clear the complete picture concerning the relationship between the salt penetration and microbial tolerance to the salt[5~7].

The penetration of Cl was remarkably difficult than that of Na in this study. This fact will be also discussed in our subsequent paper from a view point of cell permeability or salt action.

Enhancement of ^{32}P release from yeast cells by irradiation

In the hope of obtaining the evidence for increase in cell permeability, effects of NaCl concentration and irradiation on release of ^{32}P from yeast cells were investigated as well as on ^{24}Na and ^{36}Cl. The result is shown in Fig. 5.

As the active transport of phosphorus is known[8,9], the experiment was carried out at the lower temperature (3~4°), to avoid as much as possible the influence of metabolic system

5) A.K. Bruce, *J. Gen. Physiol.*, **41**, 693 (1957).
6) A. Rothstein, Radiation Research: Supplement 1, 357 (1959).
7) E.J. Conway and F. Duggan, *Biochem. J.*, **69**, 265 (1958).
8) J.W. Goodman and A. Rothstein, *Fed. Proc.*, **13**, 57 (1954).
9) A. Rothstein, *Discus. of Faraday Soc.*, No. 21, 229 (1956).

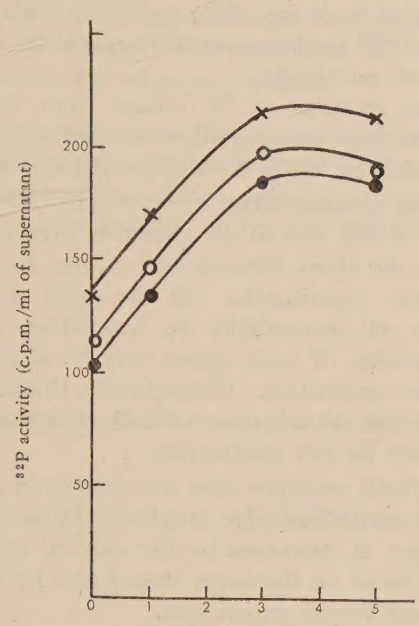

FIG. 5. Enhancement of ^{32}P Release from Yeast Cells by Irradiation.

● Unirradiated
○ Irradiated by 6×10^4r
× Irradiated by 12×10^4r
Strain, *Sacch. cerevisiae* H-336 (diploid)

within cells. The ^{32}P release increased in proportion to increasing doses in the lower concentration of NaCl, but with the higher NaCl concentrations than 3 per cent it did not increase. The 3 per cent of NaCl concentration corresponds to the lower limit of sublethal concentration for this yeast. This relationship was kept in the case of irradiated cells. The curve with control cells is only shifted upwards by irradiation and this shift was increased with increasing doses.

Thus, it was indicated that effects of the salt concentration and irradiation is additive, and this relationship may suggest the difference between the modes of actions of these two toxic factors.

These isotopic experiments using ^{24}Na, ^{36}Cl and ^{32}P support the view that the cell permeability is increased by irradiation, and that NaCl enhances this increase.

DISCUSSION

Enhancement of radiation lethal effect on microorganisms by chemical agents has been little known except oxygen and peroxides. However, the oxygen effect that enhances the lethal action of radiations has been investigated by many workers.

From a view point that oxygen effect is of indirect type, the model was presented that formation of toxic species such as HO_2 radial and H_2O_2 production by the reaction between hydrogen atom and molecular oxygen and subsequent attack to the biological targets by these radicals result in the oxygen effect[10~12]. Connecting with oxygen effect, it has been recently postulated that radiation effects can be modified by pre- or post-treatment. Moreover, it is considered that the occurrence of such modification is independent of the mode of the action of radiations, that is, in either case of direct or indirect action. Alper[13~15] discussed the modification of radiation damage of direct type effect. In contrast with the above hypothesis of indirect action, Alper considered that the effects of direct action can be also modified and postulated that oxygen may inhibit the restoration process of target damaged by direct ionization. Thus, the interest concerning radiation effect on living cells and its modification was mainly directed to the biological targets. However, the radiation chemistry suggests that radiation damages should widely occur within cells, because it has been indicated that the important biological materials are affected by ionizing radiations even with the relatively lower doses. Therefore, it would be possible that some damages in non-sensitive site as well as inactivation of targets may occur by radiation, and that some of such damages may enhance the lethal effect of a chemical agent,

10) G.E. Stapleton, *Bact. Rev.*, **19**, 26 (1955).
11) Z.M. Bacq and P. Alexander, Fundamentals of Radiobiology, pp. 209 (1955).
12) T.H. Wood, *Rev. of Modern Phys.*, **31**, 282 (1959).
13) T. Alper, Radiation Research, **5**, 573 (1956).
14) T. Alper, Advances in Radiobiology, pp. 90 (1957).
15) T. Alper et al., *Nature*, **178**, 978 (1956).

although they are not lethal in absence of the agent.

As mentioned above, it can be inferred that the radiation damages responsible for the radiation enhancement of NaCl lethal action are not restricted to the essential biological targets for radiations, because this enhancement is observable with the microorganisms which indicate the exponential survival curve. The enhancement of the cytoplasmic denaturation by radiation might be expected even with not so higher doses. In fact, the denaturation of cell components, which is probably the damage in protein and peptide fractions, was detected by infrared analysis involving the deuterium exchange method. Billen and Volkin also observed the changes in the macromolecular components of the irradiated cells by means of the analytical ultra-centrifuge and chemical analysis[16].

It is difficult to solve the question whether the denaturation of cell components observed in infrared bands associated with peptidic linkage is the indication of the cause or the result of radiation enhancement. Nevertheless there is the possibility that radiation damages of critical cell components occur and these damages become lethal for the NaCl action. The fact that influence on the rate of back-exchange was found also suggests the involvement of change in hydrogen bonding as a part of denaturation of proteins.

Furthermore, there are several evidences for increase in cell permeability by irradiation[5,6]. The facts obtained in isotopic experiments that penetration of ^{24}Na and ^{36}Cl and release of ^{32}P at the lower temperature were increased by irradiation are the strong evidence.

The experiments of ^{24}Na and ^{36}Cl penetration confirmatively indicated the increase in cell permeability by irradiation. In the case of phosphorus release from cells, as the active transport can occur, the measurements were completed in the shorter period (for 40 minutes) at the lower temperature (at 3~4°C). Subsequently, these would be, if any, little possibility that the release of ^{32}P in this experiment might be due to the cell metabolism.

In fact, amounts of ^{32}P release from yeast cells were kept constant when the cell suspensions had been left at 3~4°C for 120 minutes.

Increase in absorbance by irradiation at 260 mμ[17,18] which due to the excretion from cells supports the above view as well as the results in isotopic experiments. As the result of increase in cell permeability by irradiation, the easy invasion of toxic agents may be allowed even after irradiation. Consequently, this may result in the denaturation of cell constituents responsible for cell inactivation.

The NaCl sensitive sites are considered not to restrict to the biological targets for radiations. This view is supported by the survival curve analysis based on the target theory and by the phosphorus release experiment.

In conclusion, the possible mechanism of enhancement of radiation lethal effect by the post-NaCl treatment may involve the process of increase in cell permeability by irradiation and subsequent enhancement of the denaturation of the critical cell constituents by increasing penetration of NaCl and some radiation damages caused within cells, and thus the sensitivity of irradiated cells to NaCl may increase. However, the questions remain why the lethal salt action during irradiation is significantly more severe than after irradiation and why radioresistant tail is observed with the NaCl concentration effect curve of the salt action during irradiation. It might be inferred that the answer for the former problem lies in the view that damages of cells by irradiation are remarkably enhanced in the presence of the salt or that the rapid restoration of radiation damages associated with the lethal salt action occur.

The accounting for the latter question may be possible by the assumption of the NaCl resistant. There may be some difference of the

16) D. Billen and E. Volkin, *J. Bact.*, **67**, 191 (1954).

17) D. Billen, *Arch. Biochem. Biophys.*, **67**, 333 (1957).

18) Y. Okazawa, M. Namiki and A. Matsuyama, in preparation.

process of the salt action between during and after irradiation. These problems will be discussed in the subsequent papers.

Acknowledgements The authors wish to express their appreciation to Prof. Y. Sumiki for his guidance and Dr. S. Iida for his encouragement of the Institute of Physical and Chemical Research, and to thank Mr. K. Aizawa of the Dept. of Agricultural Chemistry, University of Tokyo, for his continuing cooperation with the infrared analysis. The authors are also very much indebted to Mr. S. Yamashita, and to Misses K. Takada, H. Ohe, and M. Yoshida, for their technical assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 443~449, 1960]

Hydrolysis of Cellulose in a Small Amount of Concentrated Sulfuric Acid

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Received November 25, 1959

An experimental procedure for preparing a homogeous mixture of cellulose and a small amount of concentrated sulfuric acid was introduced.

A diagram describing the proportions between soluble glucose-polymer, sulfuric acid and water in an equilibrium solution was constructed.

The degree of retardation of the hydrolysis of cellulose in a small amount of sulfuric acid was discussed as compared with the hydrolysis in the presence of an excess of acid.

INTRODUCTION

The wood saccharification process with concentrated sulfuric acid consists of three principal steps: the pre-hydrolysis, the main-hydrolysis and the post-hydrolysis. In the main-hydrolysis, concentrated sulfuric acid dissolves cellulose and hydrolyzes it to the short-chain glucose-polymers which are soluble in dilute sulfuric acid, and an equilibrium between soluble and insoluble polymers may be established. Concentrated sulfuric acid acts as solvent, but the solubility of cellulose in sulfuric acid of a certain concentration has not been measured and the relation of sulfuric acid concentration to the solubility has not been estimated; concentrated sulfuric acid acts as catalyst, but the hydrolysis rate of cellulose has merely been measured in the presence of a large excess of acid[1~4]. Therefore, what quantity of sulfuric acid the actual wood saccharification process requires at least is still unknown. When this problem is solved, an optimum condition of the main-hydrolysis may be determined reasonably and, in addition,

1) K. Freudenberg, W. Kuhn, W. Dürr, F. Bolz and G. Steinbrunn, *Ber.*, **63**, 1510 (1930).
2) K. Freudenberg and G. Blomqvist, *Ber.*, **68**, 2070 (1935).
3) S. Okamura, *Kōgyō Kagaku Zasshi* (*J. Soc. Chem. Ind., Japan*), **45**, 1104 (1942).
4) T. Kobayashi, *Mokuzaitōka Shingikai Hōkoku* (*Report of the Wood Saccharification Discussion Committee*), **No. 1**, 27 (1952).

TABLE I. QUANTITATIVE CHANGES OF SULFURIC ACID AND REDUCING SUGAR DURING DRYING AND HYDROLYSIS.

Before Drying			After Drying and Hydrolysis								
			P.R.S.		Total H_2SO_4						
P.R.S.	H_2SO_4	H_2O	Soluble Glucose Polymer	Insoluble Glucose Polymer	by HNO_3 Oxidation	by Hydrolysis	Free H_2SO_4	H_2O	H_2SO_4 Concn.	Recovery of Acid	Recovery of P.R.S.	Hydrolysis at 50°C
mg	mg	mg	mg	mg	mg	mg	mg	mg	%	%	%	min.
356	336	324	348	—	342	330	256	68	84	100		12
330	313	300	361	—	327	312	283	96	77	105		27
430	408	391	408	—	403	410	337	136	75	100		27
351	330	318	304	—	330	318	286	127	72	98		120
359	334	316	58	—	333	333	333	214	61	100		1080
353	332	317	—	—	324	330	333	223	60	99		2880
319	318	363	319	0	—	—	—	63	84		100	13
319	319	364	316	0	—	—	—	63	84		99	18
346	344	356	333	0	—	—	—	86	80		96	8
331	322	358	341	0	—	—	—	80	80		100	12
324	316	351	328	0	—	—	—	99	76		101	20
324	317	359	322	0	—	—	—	103	76		99	17

the interpretation of the mechanism of action of concentrated sulfuric acid on cellulose will approach completion.

However, it had been difficult to produce a homogeneous mixture of cellulose and a small amount of concentrated sulfuric acid. The authors found that by freeze-drying, dilute sulfuric acid with which cellulosic material is impregnated increases in concentration than 80%, without occurrence of decomposition of carbohydrate and sulfuric acid. Then, the present experiment now can be undertaken.

Recently, in USSR, the fact that a mechanical action accelerates the hydrolysis of wood cellulose with a small amount of concentrated sulfuric acid was reported[5]. The present study may estimate the pure chemical action of a small amount of acid on cellulose which does not work together with the mechanical action. The estimate may be useful as a control in the measurement of effectiveness of mechanical treatment on cellulose hydrolysis[6]. Moreover, this may constitute the fundamentals in the studies dealing with wood saccharification by through drying.

This paper presents the estimations of the relationship among concentrations of soluble glucose-polymer, sulfuric acid and water at equilibrium and of reaction rate constant of cellulose hydrolysis in a small amount of concentrated sulfuric acid. This paper constitutes the thirteenth of a series of articles dealing with the wood hydrolysis with strong sulfuric acid[7].

MATERIALS AND METHODS

A strip of filter paper (qualitative test use, Tôyô Roshi No. 2) ground with a grater was used as cellulosic material sample. After drying at 105°C and weighing, an adequate amount of the cellulose flakes (ca 5 g) was immersed in sufficient amount of dilute sulfuric acid (30~50%). By suction filtering through fritted-glass filter, the sample wetted by dilute sulfuric acid was obtained and the wet sample was weighed, then the proportions of the cellulose, of sulfuric acid and of water to each other were found. After being charged with the wet sample (ca. 1 g), the test tube ($12\phi \times 150$ mm, previously weighed) was weighed. Then this was put in the vacuum drying unit after cooling in the dry ice-ether

5) P.N. Odincov, A. I. Kalninsh, I.I. Beinarts and V.K. Kalnina, *Gidrolız. i Lesokhim. Prom.*, **10**, No. 8, 3 (1957).
6) H. Grohn, *J. Polymer Sci.*, **30**, 551 (1958).
7) *Ringyôshidôsho Kenkyû Hôkoku* (*Report of the Hokkaidô Forest Products Research Institute*), **No. 15**, 177 (1959).

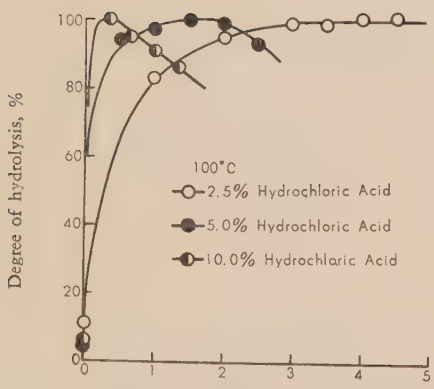


FIG. 1. Hydrolysis of Soluble Glucose-polymer in Dilute Hydrochloric Acid.

bath. The dehydration was carried out for a few hours while the weight was checked at an interval of about one hour and simultaneously the cooling was repeated. When desired amount of water had been removed, test tube was sealed in a blow lamp, being kept a vacuum, and weighed together with the waste part of the tube.

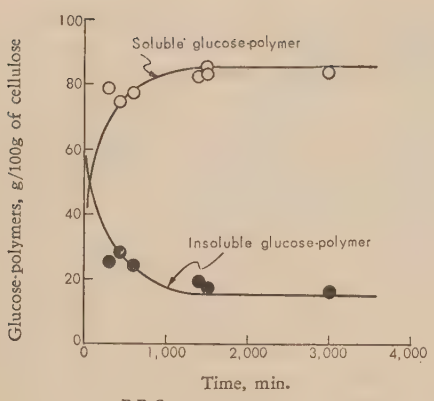
Then, the proportions of the weights of cellulose, of sulfuric acid and of water to the system after drying were found. The sealed tubes were heated in a water bath at 30, 50 and 70°C for different times. At the end of the time the tubes were immediately cooled and opened. The contents which were rinsed out with 8% sulfuric acid soluion (except the experiment of Table I) were hydrolyzed at 100°C for 2.5 hr. When some sediment appeared, it was centrifuged, washed in dilute sulfuric acid and quantitatively saccharified, using the method applied in the previous work[8,9]. The potential reducing sugar in the sediment was substituted for the estimate of the insoluble glucose-polymer; the potential reducing sugar in the supernatant and washing was substituted for the estimate of the soluble glucose-polymer. The reducing sugar was determined by Somogyi's method[10].

In the experiment of Table I, the content in the sealed tube were rinsed out with water, then the sediment was centrifuged and washed in water acidified by a drop of hydrochloric acid. To the mixture of the supernatant and the washing, hydrochloric acid was added in an amount necessary to result in 2.5% hydrochloric acid solution and it was heated at 100°C for 3 hr. This treatment was satisfactory for hydrolysis of the soluble glucose-polymer (Fig. 1). After the hydrolysis, the reducing sugar in the solution was determined and this was substituted for the soluble glucose-polymer content. In addition, four determinations of sulfuric acid were carried out: the first, sulfuric acid in the mixture of the supernatant and washing, the estimate was substituted for the free sulfuric acid content; the second, after the destruction of the organic matter sulfuric acid in the supernatant containing the washing was determined, the estimate was substituted for the total sulfuric acid (free sulfuric acid plus organic sulfuric acid combined with glucose-polymer) content; the third, sulfuric acid in the hydrolyzed supernatant containing the washing was determined, the estimate agreed with that of the second; the fourth, sulfuric acid in the sediment decomposed by nitric acid was determined, the estimate showed a trivial small amount, then sulfuric acid combined with insoluble glucose-polymer was ignored. Sulfuric acid was determined by Morgulis and Hemphill's method, iodometric micro method[11]. Combined sulfuric acid was determined according to Hoffpauir and Guthrie's method involving decomposition of the organic material with nitric acid in which a small amount of perchloric acid was added[12].

RESULTS AND DISCUSSION

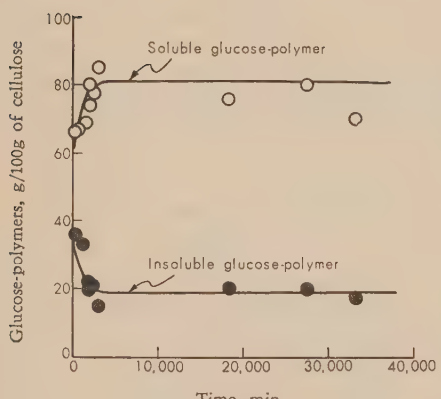
From Table I describing the quantitative change of sulfuric acid and potential reducing sugar during drying and the subsequent hydrolysis in concentrated sulfuric acid i.e. the main-hydrolysis, the following conclusion may be deduced. Sulfuric acid and potential reducing sugar which existed in the system before drying were quantitatively recovered after the drying and the hydrolysis. Although a part of sulfuric acid combined with the soluble glucose-polymer (the insoluble glucose-polymer did not combine with sulfuric acid as described above), the combined sulfuric acid was quantitatively released from by hydrolysis in dilute hydrochloric acid. These facts show that by the present procedure water in dilute sulfuric acid with which cellulose is impregnated may be evaporated without

8) T. Kobayashi and Y. Sakai, This Bulletin, **22**, 277 (1958).
9) J.F. Saeman, J.L. Bubl and E.E. Harris, *Ind. Eng. Chem., Anal. Ed.*, **17**, 35 (1945).
10) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1954).
11) S. Morgulis and M. Hemphill, *J. Biol. Chem.*,, **96**, 573 (1932).
12) C.L. Hoffpauir and J.G. Guthrie, *Ind. Eng. Chem., Anal. Ed.*, **16**, 391 (1944).



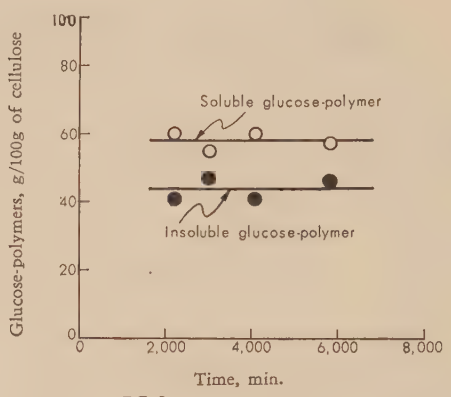
30°C	P.R.S. Insol.	P.R.S. Sol.	H_2SO_4	H_2O	$\frac{H_2SO_4}{P.R.S.}$	H_2SO_4 Concn
Initial	47	0	43	10	0.92	81
At Equil.	—	43	46	11		

FIG. 2. Hydrolysis of Cellulose in 81% Sulfuric Acid at 30°C.



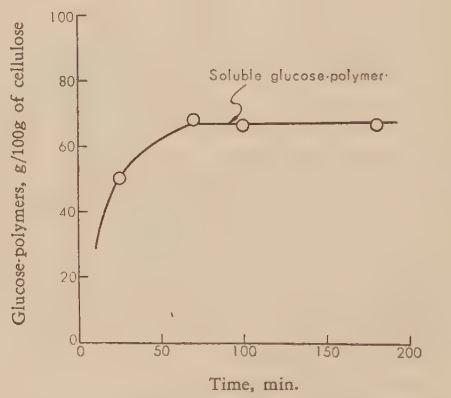
30°C	P.R.S. Insol.	P.R.S. Sol.	H_2SO_4	H_2O	$\frac{H_2SO_4}{P.R.S.}$	H_2SO_4 Concn
Initial	46	0	42	12	0.93	78
At Equil.	—	40	46	13		

FIG. 3. Hydrolysis of Cellulose in 78% Sulfuric Acid at 30°C.



30°C	P.R.S. Insol.	P.R.S. Sol.	H_2SO_4	H_2O	$\frac{H_2SO_4}{P.R.S.}$	H_2SO_4 Concn
Initial	47	0	38	15	0.82	72
At Equil.	—	34	48	19		

FIG. 4. Hydrolysis of Cellulose in 72% Sulfuric Acid at 30°C.

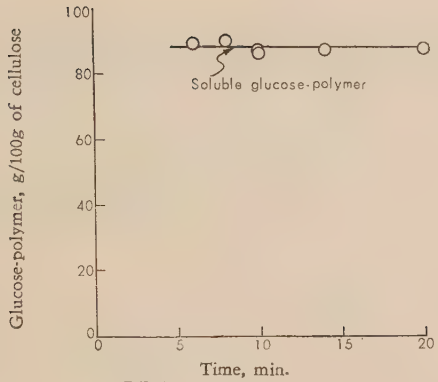


70°C	P.R.S. Insol.	P.R.S. Sol.	H_2SO_4	H_2O	$\frac{H_2SO_4}{P.R.S.}$	H_2SO_4 Concn
Initial	44	0	40	16	0.90	72
At Equil.	—	31	50	19		

FIG. 5. Hydrolysis of Cellulose in 72% Sulfuric Acid at 70°C.

accompanying with the decomposition of reducing sugar and sulfuric acid, so that loss in weight of the system can be taken to be decrease in water. Sulfuric acid concentration presented in the Table is the concentration of concentrated sulfuric acid in the system after drying; this must be called "apparent" concentration because the hydrolysis of cellulose and the reaction of sulfuric acid to cellulose which proceeds during the hydrolysis have to be accompanied with water transfer. Studies on a compound of sulfuric acid and soluble glucose polymer which is probably cellulose sulfate[13,14] produced during the main-hydrolysis will be reported elsewhere.

13) I. Kagawa, *Sen-i Gakukai Zasshi* (*J. Soc. Textile Cellulose Ind. Japan*), **1**, 677, 681 (1945).
14) G. Champetier and J. Bonnet, *Bull. soc. chim. France.*, [5], **10**, 585 (1943).



70°C	P.R.S. Insol.	P.R.S. Sol.	H_2SO_4	H_2O	$\frac{H_2SO_4}{P.R.S.}$	H_2SO_4 Conca
Initial	46	0	44	10	0.90	81
At Equil.	—	44	46	11		

FIG. 6. Hydrolysis of Cellulose in 81% Sulfuric Acid at 70°C.

A few examples of the results of the experiments in which, after increasing sulfuric acid concentration by freeze-drying, the homogeneous mixture of cellulose and acid were subjected to hydrolysis are given in Figs. 2 to 6. At an early stage in reaction, cellulose was hydrolyzed to soluble glucose-polymer rapidly and the hydrolysis approached slowly an equilibrium in which soluble glucose-polymer did not increase and insoluble glucose-polymer did not decrease and when reaction time was extremely prolonged the sum of two polymers decreased. In Fig. 2, for example, when the mixture of the composition indicated by "Initial" was subjected to hydrolysis, 85% of cellulose (i.e. insol. P.R.S.*) converted to soluble glucose-polymer (i.e. sol. P.R.S.**) and the hydrolysis reached the equi-

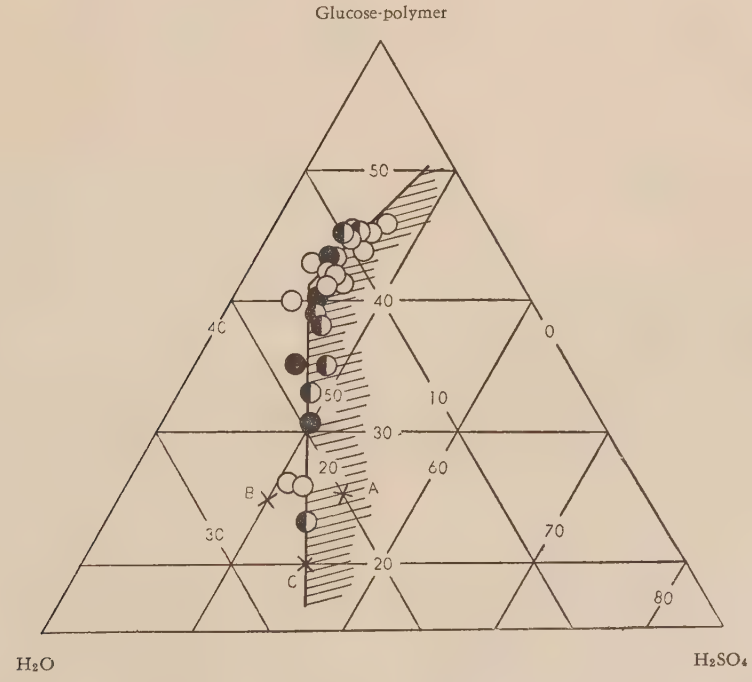


FIG. 7. Equilibrium of Hydrolysis of Cellulose in a Small Amount of Concentrated Sulfuric Acid.

○ 30°C ◑ 50°C ● 70°C

* insoluble potential reducing sugar
** soluble potential reducing sugar

TABLE II. COMPARISON OF T_{equil} AND T_{wh} AT 30°C

"Apparent" Sulfuric Acid Strength %	H_0	T_{equil} min.	T_{wh} min.	T_{equil}/T_{wh}
87	−7.92	500	0.08	6200
85	−7.62	1000	0.2	5000
81	−6.98	1500	0.7	2100
80	−6.84	3000	1.0	3000
79	−6.48	3500	2.3	1500
78	−6.38	4000	2.9	1400
76	−6.28	2200	3.8	600

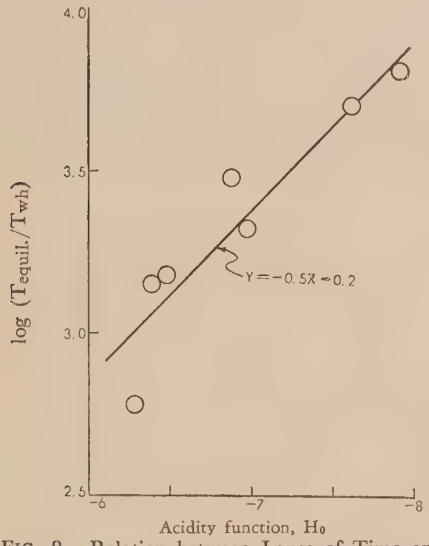


FIG. 8. Relation between Lapse of Time and Acid Strength in Hydrolysis of Cellulose in a Small Amount of Sulfuric Acid (H_2SO_4/cellulose =0.9/1.0) at 30°C.

librium in which the proportion between soluble glucose-polymer, sulfuric acid and water was given by "At Equil".

Thus twenty five equilibrium data were determined and these were plotted on the diagram in Fig. 7. Two straight lines which fit the data indicate the equilibrium curve and the hatched region shows the range in which concentrated sulfuric acid is not saturated by soluble glucose-polymer. For example, point A describes that the mixture of 55 parts of sulfuric acid and 20 parts of water can convert more than 25 parts of cellulose (as potential reducing sugar) into soluble glucose-polymer that is 100 g of 73% sulfuric acid can hydrolyze more than 33 g of cellulose (as potential reducing sugar) to soluble glucose-polymer; point B, 100 g of 67% sulfuric acid can not convert 33 g of cellulose (as potential reducing sugar) into soluble glucose-polymer; point C, 100 g of 69% sulfuric acid can convert 31 g of cellulose (as potential reducing sugar) into soluble glucose-polymer.

The equilibrium curve which is extrapolated to the line indicating that no sugar is contained passes through a point representing 65% sulfuric acid concentration. This observation agrees with the fact that cellulose dissolution into sulfuric acid begins at concentration of 10.5 mole per liter[15].

From such figures as Figs. 2 and 3, the retention times that is necessary to be established the hydrolysis equilibrium, may be observed (these times are expressed by "T_{equil}'s," here). When the hydrolysis proceeds completely in a large excess of acid, the retention time is expressed by "whole time, T_{wh}"[4]. In Table II, the retention time, "T_{equil}", is compared with the "whole time, T_{wh}" in order to compare the hydrolysis rate of cellulose in a small amount of concentrated sulfuric acid (H_2SO_4/cellulose=0.9/1.0) at 30°C with that in a large excess of acid. "Whole time, T_{wh}" is calculated from

$$T_{wh}=0.45/[4.75\times10^{11}C^{-1.02}\exp\{-26400/(RT)\}]^{16)},$$

where $\log C=H_0$.

Figure 8 shows that the lapse of time in hydrolysis in a small amount of sulfuric acid increases with increased acid strength (apparent) in comparison with the lapse of time in complete hydrolysis in a large excess of acid. Then, the relation of $\log(T_{equil}/T_{wh})$ and H_0 may be expressed by the following equation,

$$\log(T_{equil}/T_{wh})=-0.5H_0-0.2.$$

Then, the equation describing the lapse of time which is necessary to be established the

15) W.W. Pigman, "Chemistry of the Carbohydrate", New York, Academic Press, 1948, p. 545.

16) T. Kobayashi and Y. Sakai, "Kôbo Riyô Kôgyô", T. Asai ed., Tôkyô Kyôritsu Shuppansha, "Biseibutsu Kôgaku Kôza", 1957, Vol. IV, pp. 195~197.

equilibrium in the hydrolysis of cellulose in a small amount of sulfuric acid (H_2SO_4/cellulose =0.9/1.0) at 30°C is given by the following equation,

$$T_{equil}=0.45/[4.75\times10^{11.2+0.5H_0-1.02}\exp\{-26400/(RT)\}],$$

where $\log C=H_0$.

Acknowledgement For their helpful suggestions the authors wish to thank the members of the Wood Saccharification Committee. The authors are grateful to Miss W. Kumagai for her assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 449~458, 1960]

On the Mechanism of L-Prolyl Diketopiperazine Formation by *Streptomyces**

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Received November 28, 1959

Since L-prolyl diketopiperazines, L-prolyl-L-valine anhydride and L-leucyl-L-proline anhydride, had been isolated from the culture filtrate of *Streptomyces* sp. S-580, the mechanism of L-prolyl diketopiperazine formation by *Streptomyces* has been studied. These two L-prolyl diketopiperazines were not formed from their constituent amino acids incubated with intact cell or cell free homogenate of this strain in buffered salt solution containing energy source. However, from milk casein, poly peptone or gelatin, the former two were components of the culture medium of this strain, hydrolyzed with the pure streptomyces-protease, these L-prolyl diketopiperazines were obtained (only from gelatin, glycyl-L-proline anhydride were obtained in addition to these two). Furthermore, in hydrolysis of some synthetic L-prolyl peptides with this enzyme, L-prolyl diketopiperazine formation were also studied, and as the result, glycyl-L-proline anhydride was obtained from glycyl-L-prolyl-L-leucine but no L-prolyl diketopiperazine was formed from L-prolyl-L-leucyl-glycine. From these evidences, the possible route of L-prolyl diketopiperazine formation by *Streptomyces* has been discussed.

INTRODUCTION

In the previous papers[1,2], it has been reported that two L-prolyl diketopiperazines (PDKP), L-prolyl-L-valine anhydride (PVA) and L-leucyl-L-proline anhydride (LPA), have been isolated from the culture filtrate of *Streptomyces* sp. S-580. In 1951, J. L. Johnson et al[3]. had isolated LPA from the culture filtrate of *Streptomyces* sp, *St. griseus* or *Aspergillus fumigatus*. Recently, PVA and LPA have been isolated from the culture filtrate of *Penicillium ochraceus* by H. Kodaira[4], furthermore, these two and L-phenyl-

* Presented at the Meeting of the Agricultural Chemical Society of Japan held in Tokyo, February 28, 1959 and at the Annual Meeting of the Biochemical Society of Japan held in Osaka, November 1, 1959.

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1) Y. Koaze, This Bulletin, **22**, 98 (1958).

2) Y. Koaze, *ibid.*, **24**, 530 (1960).

3) I.L. Johnson et al., *J.A.C.S.*, **73**, 2946 (1951).

4) H. Kodaira, a personal communication.

alanyl-L-proline anhydride have been isolated from the culture filtrate of *Rosellinia necatrix* by Yu. Chen[5]. On the other hand, in 1935, LPA had been isolated from hog adrenal cortex extract[6] and previously in 1906, glycyl-L-proline anhydride (GPA) had been isolated from gelatin hydrolyzed with trypsin by P.A. Levene and W.A. Beatty[7], and, in 1923, LPA had been isolated from gliadin hydrolyzed with pancreatin by E. Abderhalden[8]. However, on the mechanism of PDKP formation by microorganism, even by any other living organism, no report has appeared yet. Then in this paper, formation of PVA and LPA by *St.* sp. S-580 has been studied on incubation of intact cell or cell free homogenate of this strain with constituent amino acids of these PDKP, and on hydrolysis of proteinous substances which have been components of the culture medium of this strain with the pure streptomyces-protease, furthermore PDKP formation from straight-chain L-prolyl peptides hydrolyzed with this enzyme has been studied. And from these evidences, the possible route of PDKP formation by *Streptomyces* has been discussed.

EXPERIMENTAL AND RESULTS

(I) Incubation of intact cell or cell free homogenate of *St.* sp. S-580 with constituent amino acids of PDKP.

(1) Experiment on intact cell.

In shaking culture, *St.* sp. S-580 was grown on bouillon-glucose medium for 48 hours at 27°C. The cells were harvested on the centrifuge, 9000 r.p.m. for 10 minutes, and washed twice with buffered salt solution (M/15 phosphate buffer pH 7.4 containing 20 mM KCl, 10 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 10 μM $ZnSO_4$). The washed cells were shaken for 2 hours at 30°C in buffered salt solution and collected on the centrifuge again. Ten grams of the cells were suspended in 100 ml buffered salt solution containing 1% glucose with or without 575.7 mg L-proline and 585.8 mg L-valine, or 575.7 mg L-proline and 655.9 mg L-leucine. Suspensions were shaken for 3 hours at 30°C in 500 ml flask. After shaking, in each sample, the cells were removed on the centrifuge and the supernatant was freeze-dried. The dried matter was suspended in 50 ml chloroform and extracted for 3 days at 20~25°C shaking occasionally. After extraction, the insoluble matter was filtered off and the solvent was evaporated under reduced pressure. The concentrate was dissolved in 20 ml chloroform again and decolored with 1% active carbon for 20 minutes at 20~25°C agitating continuously. After decoloration, the active carbon was filtered off and the solvent was evaporated under reduced pressure. Then on the concentrate obtained, detection was carried out whether PDKP were contained. For this purpose, the paper chromatography described below was employed. By the usual way, the each condensate was applied to the filter paper of Tōyōroshi No. 50 and ascended with water-saturated *tert*-amylalcohol for 24~36 hours at 20°C (for control, authentic PVA and LPA were also applied to the same filter paper). After removing the solvent, the filter paper was sprayed with 1% ninhydrin in water-saturated *n*-butanol. Next, the same paper was exposed to Cl_2 gas for 10 minutes and the excess Cl_2 was removed, then sprayed with a 1% soluble starch - 1% KI solution (Rydon-Smith method[9]). In general, PDKP are not developed with ninhydrin but appear as blue spots through Rydon-Smith method. As the result, controls, authentic PVA and LPA, were developed to have R_F of 0.87 and 0.93 (sensitivities have been 20 mcg and 10 mcg individually), however on all spots of concentrates, PDKP were not detected. Therefore, it has been presumed that PVA and LPA have not been formed from their constituent amino acids incubated with intact cell of *St.* sp. S-580 under the condition described above.

(2) Experiment on cell free homogenate.

Under the same conditions described above, *St.* sp. S-580 was grown for 72 hours. The cells were harvested and washed through the same method described above. Fourteen grams of the washed cells were ground with 28 g alumina (aluminium oxide E.P., Kosō Chemicals Co.) in a mortar and homogenized in 25 ml buffered salt solution for 30 minutes at 2~4°C. Then the homogenate was cleared of abrasives and cells unground by a low-speed centrifugation, 6000 r.p.m. for 30 minutes. The resultant suspension was divided into two equal portions and incubated with or without 23.0 mg L-proline and 23.4 mg L-valine in 20 ml buffered salt solution containing 124.6 mg adenosine tri-phosphate (tri-sodium salt) for 60 minutes at 38°C individually. After incuba-

5) Yu. Chen, a personal communication.
6) O. Wintersteiner and J.J. Pfiffner, *J.B.C.*, **111**, 599 (1935).
7) P.A. Levene und W.A. Beatty, *Ber.*, **39**, 2060 (1906).
8) E. Abderhalden, *Z. physiol. Chem.*, **128**, 119 (1923).
9) H.N. Rydon and P.W.G. Smith, *Nature*, **169**, 922 (1952).

tion, through the same procedure described above, each sample was freeze-dried, extracted with chloroform and on the concentrate obtained, by the paper chromatography described above, detection was carried out whether PDKP were contained. As the result, the control, authentic PVA was developed, however on the spot of each sample, PDKP were not detected. Therefore, it has been presumed that PVA has not been formed from its constituent amino acids incubated with cell free homogenate of *St.* sp. S-580 under the condition described above.

(II) Hydrolysis of milk casein, poly peptone and gelatin with the pure streptomyces-protease.

(1) Hydrolysis of milk casein.

Fifty grams of milk casein (Junsei Pure Chemicals & Co., Ltd.), a component of the culture medium of this strain, were dissolved in 1000 ml M/10 sodium acetate solution containing M/50 calcium acetate and then incubated with 250 mg the pure streptomyces-protease (obtained from the culture filtrate of a streptomycin-producing *St. griseus* by M. Nomoto et al.[10], Institute of Physical and Chemical Research) for 72 hours at 30°C (50 ml toluene was added to this solution for prevention of putrefaction). After incubation, the reaction mixture was concentrated to about 100 ml under reduced pressure on the water bath (below 40°C) and then freeze-dried. The dried matter was ground in a mortar and suspended in 300 ml chloroform, then heated in a flask with reflex-condensor on the water bath (at 60±1°C) for 20 hours. The insoluble matter was filtered off and by evaporating the solvent under reduced pressure, 2.66 g solid matter was obtained. By a column chromatography using active carbon (Wakō Pure Chemical Industries, Ltd.) described below, PDKP contained in the crude extract were isolated. By the usual way, 26.6 g active carbon, suspended in a mix-solvent of chloroform : ether = 1 : 1, was introduced into a glass tube with diameter 2.5 cm and the crude extract dissolved in 10 ml chloroform was charged into this column and eluted with a sufficient volume of the mix-solvent described above at the flow rate of 1.0~2.0 ml/min. Each 10 ml of eluant was collected into test tube and on each tube, the paper chromatography described above was carried out for detection of PDKP. Fractions of PDKP obtained were gathered and the solvent was evaporated under reduced pressure and the resultant residue washed with petroleum ether, then 173.9 mg pure PDKP was obtained (yield 0.35%). From the preparation without this enzyme, no PDKP was obtained under the same condition described above. By paper chromatography described above, PDKP obtained was presumed to be a mixture of PVA and LPA as shown in Fig. 1 and on comparison of infra-red spectrum between PDKP obtained and a mixture of authentic PVA and LPA as shown in Fig. 2, the former was identified with the latter. Next, PDKP obtained was hydrolyzed with 6 N HCl and the each constituent amino acid, L-leucine, L-proline and L-valine, was determined through a bioassay method by the usual way, and as the result, it was demonstrated that PVA and LPA were mixed in the PDKP obtained in the molar ratio of 100 : 98. On the other hand the substrate, milk casein, was also hydrolyzed with 6 N HCl and the constituent L-proline was determined and then the yield of PDKP-L-proline against substrate-L-proline was computed to 2.03%.

(2) Hydrolysis of poly peptone.

Fifty grams of poly peptone (Wakō Pure Chemical Industries, Ltd.) a component of the culture medium

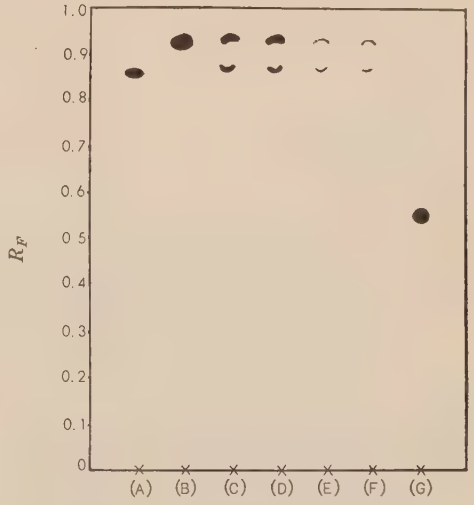


FIG. 1. Paper Chromatograph of PDKP Isolated from Milk Casein, Poly Peptone and Gelatin Hydrolyzed with the Pure Streptomyces-protease.

(A) authentic L-prolyl-L-valine anhydride (control).
(B) authentic L-leucyl-L-proline anhydride (〃).
(C) PDKP isolated from milk casein hydrolyzed with the pure streptomyces-protease
(D) 〃 poly peptone 〃
(E) 〃 〃 treated without this enzyme.
(F) PDKP fraction G-I isolated from gelatin hydrolyzed with this enzyme.
(G) 〃 G-II 〃

10) M. Nomoto and Y. Narahashi, *Rept. Inst. Phys. Chem. Research* (Japan), **34**, No. 6, 381, 393, 399 (1958).

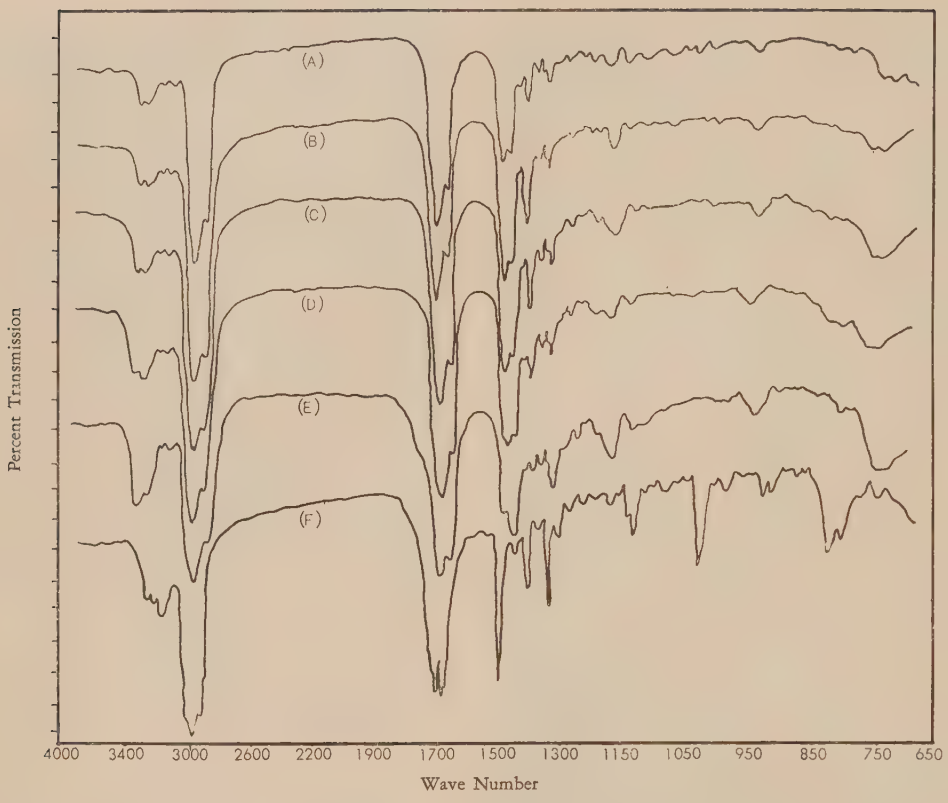


FIG. 2. Infra-red Spectra of PDKP Isolated from Milk Casein, Poly Peptone and Gelatin Hydrolyzed with the Pure Streptomyces-protease. (in Nujol Mull)

(A) a mixture of authentic L-prolyl-L-valine anhydride and L-leucyl-L-proline anhydride (control).
(B) PDKP isolated from milk casein hydrolyzed with the pure streptomyces-protease.
(C) 〃 poly peptone 〃
(D) 〃 〃 treated without this enzyme.
(E) PDKP fraction G-I isolated from gelatin hydrolyzed with this enzyme.
(F) 〃 G-II 〃

of this strain, were previusoly extracted with chloroform* and then the resultant residue was dissolved in 1000 ml sodium acetate solution described above and divided into two equal portions and incubated with or without 125 mg streptomyces-protease under the same condition as described above individually. After incubation, under the same procedure mentioned above, reaction mixtures were concentrated, freeze-dried and extracted with chloroform. 426 mg (with enzyme) and 358 mg (without enzyme) crude extract were obtained individually, and purified further with active carbon-column chromatography (4.3 g and 3.6 g active carbon were used individually) and 55.1 mg and 9.3 mg pure PDKD was obtained individually, and accordingly the net-PDKP formation was 45.8 mg (yield 0.18% and the yield of PDKP-L-proline against substrate-L-proline was 1.08%). As shown in Fig. 1 and 2, these PDKP obtained were a mixture of PVA and LPA, and in PDKP obtained with enzyme molar ratio of PVA to LPA was 100 : 98.

(3) Hydrolysis of gelatin.

Fifty grams of gelatin (Junsei Pure Chemicals & Co., Ltd.) were dissolved in 1000 ml sodium acetate solution described above and incubated with 250 mg streptomyces-protease under the same condition as described above.

* From this chloroform extract two PDKP, LPA and PVA, were also isolated. Accordingly these PDKP have been presumed to be formed from milk casein hydrolyzed with trypsin, because poly peptone used in this experiment is a hydrolysate of milk casein with pancreas.

Table I. Comparison of Properties between Glycyl-L-proline Anhydride Isolated from Gelatin Hydrolyzed with the Pure Streptomyces-protease, Synthesized by Fischer et al. and Isolated by Levene et al.

		isolated by the author	calculated for $C_7H_{10}O_2N_2$	synthesized by Fischer et al.	isolated by Levene et al.
results of elementary analysis	C%	55.00	54.53	54.49	53.93
	H%	6.47	6.54	6.51	6.66
	N%	17.65	18.17	18.01	18.51
molecular weight		144*	154.17	no description	no description
melting point		209~211°C	—	209°C	182~185°C
$[\alpha]_D$		−218.9° (17°C, in water)	—	−206.5° (20°C, in water)	no description
ninhydrin reaction		negative	—	—	negative

* measured by the micro Rast method dissolving in camphor.

Table II. Results on Hydrolysis of Milk Casein, Poly Peptone and Gelatin with the Pure Streptomyces-protease.

	milk casein	poly peptone	gelatin
weights of substrate	50 g	25 g	50 g
// crude extract	2660 mg	426 mg (358 mg)*	517 mg
// total PDKP	173.9 mg	55.1 mg (9.3 mg) net 45.8 mg	154.4 mg
yield of total PDKP against substrate	0.35%	0.18%	0.31%
yield of PDKP-L-proline against substrate-L-proline	2.03%	1.08%	1.52%
kinds of PDKP obtained	PVA and LPA	PVA and LPA	PVA, LPA and GPA
molar ratio of a mixture	PVA : LPA = 100 : 98	PVA : LPA = 100 : 98	PVA : LPA : GPA = 100 : 224 : 8582

* figures in parentheses show values obtained from treatments without enzyme.

After incubation, under the same procedure mentioned above, the reaction mixture was concentrated, freeze-dried and extracted with chloroform and 517 mg the crude extract was obtained, and purified further with active carbon-column chromatography (5.2 g active carbon was used) and then 154.4 mg pure PDKP was obtained (yield 0.31% and the yield of PDKP-L-proline against substrate-L-proline was 1.52%). From the preparation without this enzyme, no PDKP was obtained under the same condition described above. The PDKP obtained was separated to two fractions, named G-I (7.4 mg) and G-II (147.0 mg). As shown in Fig. 1 and 2, fraction G-I was a mixture of PVA and LPA having the molar ratio of 100 : 224. On the other hand, fraction G-II was obviously distinguished from these two PDKP as shown in Fig. 1 and 2. This one has been proved by hydrolysis (glycine and proline were obtained) and from its properties, as shown in Table I to be glycyl-L-proline anhydride (GPA). Therefore, it has been manifested that the majority of PDKP obtained from gelatin with this enzyme was GPA.

In Table II, these results obtained as above-mentioned have been summarized.

(III) Hydrolysis of some synthetic L-prolyl peptides with the pure streptomyces-protease.

(1) Hydrolysis of L-prolyl-L-leucyl-glycine and L-prolyl-L-leucine.

Twenty mg of L-prolyl-L-leucyl-glycine (PLG, synthesized by S. Sakakibara, Institute for Protein Research, Univ. of Osaka) and L-prolyl-L-leucine (PL, synthesized by the author) were hydrolyzed with 200 mcg of the streptomyces-protease described above in 4 ml sodium acetate solution mentioned above adding with 0.5 ml toluene under the same conditions described above. After incubation, each reaction mixture was concentrated to dryness on the water bath (below 40°C) under reduced pressure. The dried matter was suspended in 20 ml chloroform and then heated in a flask with reflex-condensor on the water bath (at 60±1°C) for 60 minutes. The insoluble matter was filtered off and the solvent was evapolated under reduced pressure. On the concentrate obtained, by the paper chromatography described above,

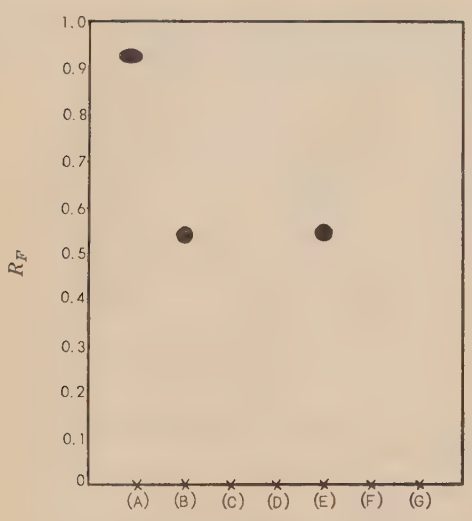


FIG. 3. Paper Chromatograph of Chloroform-extracts from Reaction Mixtures on Hydrolysis of L-Prolyl Peptides with the Pure Streptomyces-protease.

(A) authentic L-leucyl-L-proline anhydride (control).
(B) authentic glycyl-L-proline anhydride (〃).
(C) the chloroform-extract from reaction mixture on hydrolysis of L-prolyl-L-leucyl-glycine with the pure streptomyces-protease.
(D) 〃 of L-prolyl-L-leucine with this enzyme.
(E) 〃 of glycyl-L-prolyl-L-leucine with this enzyme.
(F) 〃 of glycyl-L-proline with this enzyme.
(G) the chloroform-extract from reaction mixture on treatment of glycyl-L-proline without this enzyme.

detection was carried out whether PDKP were obtained. As the result, shown in Fig. 3, no PDKP was detected. On the other hand, by the paper chromatography employing for detection of amino acids, it has been obvious that these two L-prolyl peptides have been hydrolyzed to constitutent amino acids individually with this enzyme under conditions described above.

(2) Hydrolysis of glycyl-L-prolyl-L-leucine and glycyl-L-proline.

One hundred mg glycyl-L-prolyl-L-leucine (GPL, synthesized also by S. Sakakibara) was hydrolyzed with 1 mg this enzyme in 10 ml sodium acetate solution described above adding with 2 ml toluene under the same conditions mentioned above. After incubation, under the same procedure described above, the reaction mixture was concentrated to dryness, extracted with chloroform and then the crude extract obtained was examined for PDKP detection by the paper chromatography described above. As the result, a spot was developed through-Rydon-Smith method at the same R_F of GPA (isolated from the hydrolyzate of gelatin with this enzyme as mentioned above) as shown in Fig. 3. The PDKP obtained was isolated by the paper chromatography described above eluting with a sufficient volume of hot methanol twice and purified with ether and 3.1 mg pure crystal were obtained (yield 3.1%, and the yield of L-proline in the PDKP against L-proline in this tri-peptide was 5.2%). The mixed melting point of this substance with GPA described above showed no depression and the infra-red spectrum of this substance coincide with that of GPA as shown in Fig. 4. As the result, this substance has been identified with GPA. Under the same procedure without this enzyme, no GPA was obtained from this tri-peptide.

On the other hand, ten mg glycyl-L-proline* (GP, General Biochemical INC.) was hydrolyzed with and without 100 mcg this enzyme in 1 ml sodium acetate solution described above adding with 0.5 ml toluene under the same conditions mentioned above. After incubation, under the same procedure described above, reaction mixtures were concentrated to dryness, extracted with chloroform and then crude extracts obtained were examined for PDKP detection by the paper chromatography described above. As the result, both spots, samples with and without enzyme, were developed through Rydon-Smith method at the same R_F of GPA isolated as above mentioned (Fig. 3). Then, on these three samples (GPL with enzyme, GP with and without enzyme), quantitative determination of GPA formed was carried out as follows. These samples were ascended on a paper by the paper chromatography described above and spots developed through Rydon-Smith method at the same R_F of GPA were cut out of the paper and extracted with 10 ml methanol in flask with reflex-condensor twice on the water bath (at 60±1°C) for 10 minutes. These methanol solutions obtained as above mentioned were concentrated under reduced pressure, resultant extracts were hydrolyzed with 6 N HCl at 120°C for 18 hours by the usual way. On hydrolysis, GPA is decomposed to glycine and L-proline, and then through the determination of glycine obtained by Moore-Stein method[11], the quantity of GPA is calculated out. (For control, a suitable quantity of GPA, 250 mcg in this case, was treated under the same procedure described above and as the result, the quantity obtained through the method described above was 226 mcg, yield 90.4%.) As shown in Table III, yields of GPA from GPL with

* This GP sample contained GPA as a impurity, therefore this sample was extracted with chloroform before experiment.

11) S. Moore and W.H. Stein, *J.B.C.*, **211**, 907 (1954).

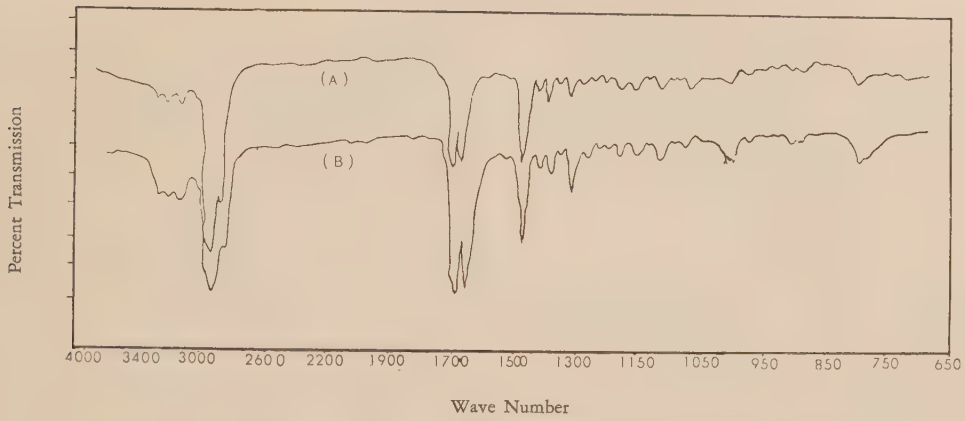


FIG. 4. Infra-red Spectrum of Glycyl-L-proline Anhydride Formed from Glycyl-L-prolyl-L-leucine Hydrolyzed with the Pure Streptomyces-protease. (in Nujol Mull)

(A) glycyl-L-proline anhydride formed from glycyl-L-prolyl-L-leucine hydrolyzed with the pure streptomyces-protease.

(B) " isolated from gelatin hydrolyzed with this enzyme (control).

TABLE III. COMPARISON OF THE YIELD BETWEEN GLYCYL-L-PROLINE ANHYDRIDE FORMED FROM GLYCYL-L-PROLYL-L-LEUCINE HYDROLYZED WITH THE PURE STREPTOMYCES-PROTEASE AND FROM GLYCYL-L-PROLINE TREATED WITH OR WITHOUT THIS ENZYME.

	glycyl-L-prolyl-L-leucine with enzyme	glycyl-L-proline with enzyme	glycyl-L-proline without enzyme
weights of substrate	100 mg	10 mg	10 mg
weights of GPA obtained	4930 mcg	132 mcg	145 mcg
yield of GPA obtained	4.93% (8.17%)*	1.32%	1.45%
comparison of yields	100	16	18

* calculated of GPA obtained against glycyl-L-proline bond in this tri-peptide used.

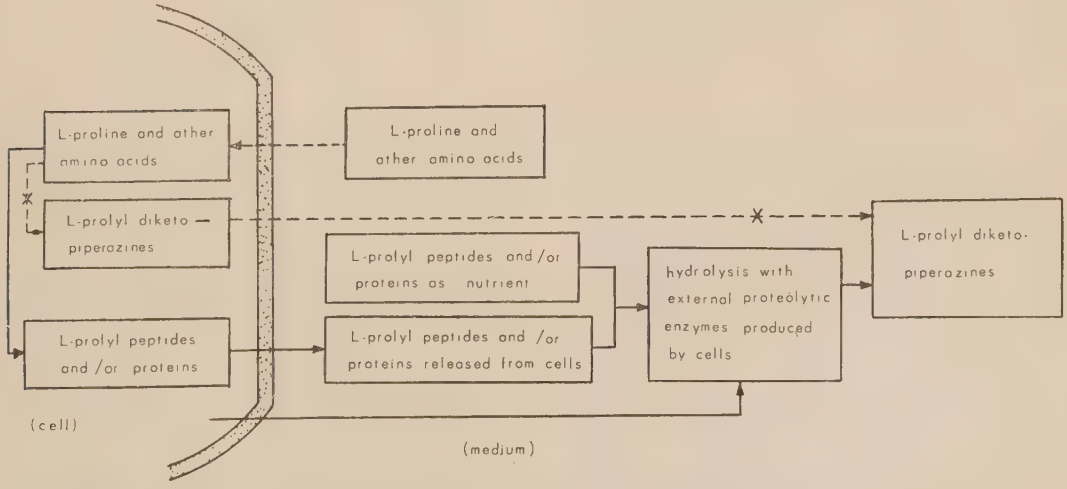


FIG. 5. Outlines of the Possible Pathway of L-Prolyl Diketopiperazine Formation by *Streptomyces*.

--- rout A —— rout B

enzyme, GP with and without enzyme were 8.17% (against GP-peptide bond in GPL), 1.32% and 1.45% individually. Therefore, it has been obvious that GPA has been formed from GPL hydrolyzed with the streptomyces-protease and furthermore no enzymic GPA formation from GP was recognized, however, the yield in this case was less then 20% against the yield in the case of GPL with enzyme under the conditions described above.

DISCUSSION

From these evidences described above, the most possible pathway of L-prolyl diketopiperazine formation by *Streptomyces* has been discussed as follows.

(1) In incubation of the intact cell or the cell free homogenate of *Streptomyces* sp. S-580 with L-proline and the other amino acid, L-leucine or L-valine, in the buffered salt solution containing energy source, neither LPA nor PVA which had been isolated from the culture filtrate of this strain, was formed. However, on hydrolysis of milk casein or poly peptone, a component of the culture medium of this strain, with the pure streptomyces-protease, LPA and PVA were obtained obviously and furthermore on hydrolysis of gelatin with this enzyme, in addition to these two PDKP, GPA, which had been isolated from gelatin hydrolyzed with trypsin by Levene et al.[7] in 1923, was also obtained. Therefore, as shown in Fig. 5, these two PDKP isolated from the culture filtrate of this strain have not been synthesized from free amino acids which were in the cell as pool and/or up-take from the culture medium (route-A), but formed from L-prolyl peptides and/or proteins which were in the culture medium added as nutrients or released from cells, hydrolyzed with the external proteolytic enzyme produced by this strain in the culture filtrate (route-B).

(2) Since GPA has been formed from GPL hydrolyzed with the enzyme described above, it has been presumed that some PDKP have been formed from some straight-chain L-prolyl peptides hydrolyzed with some proteolytic enzymes. Accordingly on the route-B described above, it has been presumed that PDKP have been formed from straight-chain L-prolyl peptide bonds in L-prolyl peptides and/or proteins hydrolyzed with this enzyme. On the other hand, it has been also presumed that PDKP obtained have been nothing but released from bound PDKP with L-prolyl peptides and/or proteins hydrolyzed with this enzyme as follows.

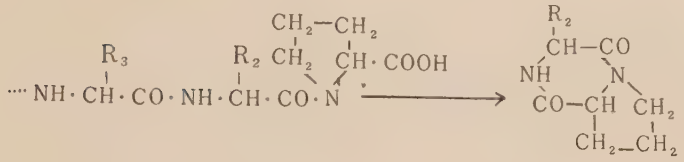
However, the enzyme used in the experiment described above has been next to impossible to hydrolyze glycyl-L-proline to glycine and L-proline[12,13] under the usual condition, and then it has been presumed that –CO-N< bond has been hardly decomposed with this enzyme, accordingly this presumption has also hardly held true.

(3) As mentioned above, PDKP have been formed from straight-chain L-prolyl peptides hydrolyzed with the enzyme described above and then three pathway have been presumed as follows.

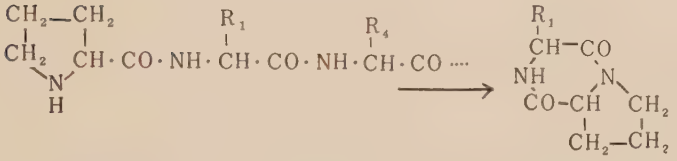
Formation of diketopiperazine-ring is considered as formation of peptide bond and in this case, as usually, activation of carbonyl group must occur. Accordingly it has been presumed that the reaction formulated as (I) has been excluded because there is no carbonyl group which must be activated for form PDKP. Further-

12) S. Sasakawa and K. Satake, *J. Biochem.* (*Japan*), **45**, 867 (1958).

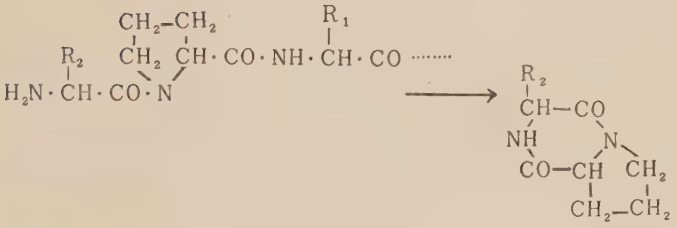
13) M. Nomoto et al., *Rept. Inst. Phys. Chem. Research* (*Japan*), **35**, No. 4, 261 (1959).



(I)



(II)



(III)

more, since no PDKP were formed from PLG or PL, L-proline was located at amino terminal in these peptides, hydrolyzed with this enzyme (these L-prolyl peptides were decomposed to individual amino acids), it has been presumed that the reaction formulated as (II) has been also excluded. Therefore, from the evidence that GPA was formed from GPL, L-proline was located at middle in this peptide, hydrolyzed with this enzyme, it has been presumed that PDKP has been formed in the reaction formulated as (III). On the other hand, on the formation of GPA from GPL with this enzyme, following two reactions have been presumed.

$$\text{GPL} \xrightarrow{\text{enzymic}} \text{GPA} \qquad \text{(IV)}$$

$$\text{GPL} \xrightarrow{\text{enzymic}} \text{GP} \xrightarrow{\text{spontaneous}} \text{GPA} \qquad \text{(V)}$$

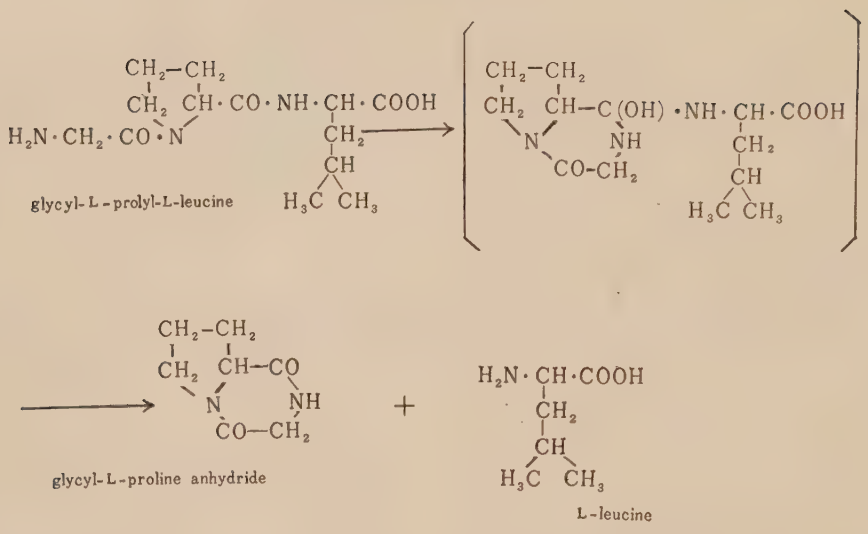
From the evidence that the yield of GPA formed from GP without enzyme was less than 20% against the yield of GPA formed from GPL with enzyme, the main pathway of GPL to GPA has been presumed to be the reaction (IV).

In 1950, J. S. Fruton[14] proposed a transpeptidation reaction on hydrolysis of benzoyl-L-tyrosyl-glycine amide with chymotrypsin. According to Fruton's mechanism, the reaction of GPL to GPA has been presumed to be formulated as in the diagram below, the postulated intermediate being enclosed in brackets.

And then this pathway has been presumed to be an internal transpeptidation reaction.

Acknowledgements The author wishes to express his sincere thanks to Professor Emeritus K. Sakaguchi and Prof. K. Arima, Univ. of Tokyo for their constant guidances throughout this work, and also to Prof. Y. Sumiki and Prof. E. Ando, Univ. of Tokyo for their kind advices and suggestions extended throughout this work. Thanks are also due to Prof. S.

14) R.B. Johnston, M.J. Mycek and J.S. Fruton, *J.B.C.*, **187**, 205 (1950).



Akabori, Univ. of Osaka for his generous supply of L-prolyl tri-peptides and to Mr. M. Nomoto, Inst. of Physical and Chemical Research for his kind supply of the pure streptomyces-protease. The elementary analysis, the microbioassay and and infra-red spectra were carried out at the Inst. of Applied Microbiology and this department.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 459~463, 1960]

Studies on the Syntheses of the Pyrethrin Analogues and their Biological Activities

Part I. Allethronyl Esters of *Cyclo*propanecarboxylic Acids

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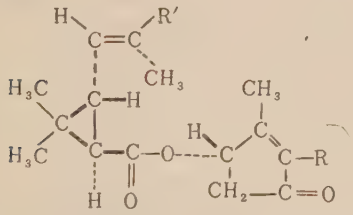
Received December 5, 1959

Some new allethronyl esters of *cyclo*propanecarboxylic acids were prepared, and their insecticidal activities were tested against common housefly. Allethronyl esters of *cyclo*propanecarboxylic acids having 3,4-methylenedioxyphenyl group on the *cyclo*propane ring were more or less toxic. Among them, allethronyl 2,2-dimethyl-3-(3′,4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate was found to be more toxic than *α-dl-trans*-allethrin, and the calculated relative effectiveness were 1.48 and 1.21 on the mortality and knock-down activity respectively as compared with *α-dl-trans*-allethrin.

The active principles of pyrethrum flower are known to be a mixture of four keto-esters, generally referred to as the "Pyrethrins". Four naturally occurring pyrethrins are recognized to be represented by the following general fomulae:



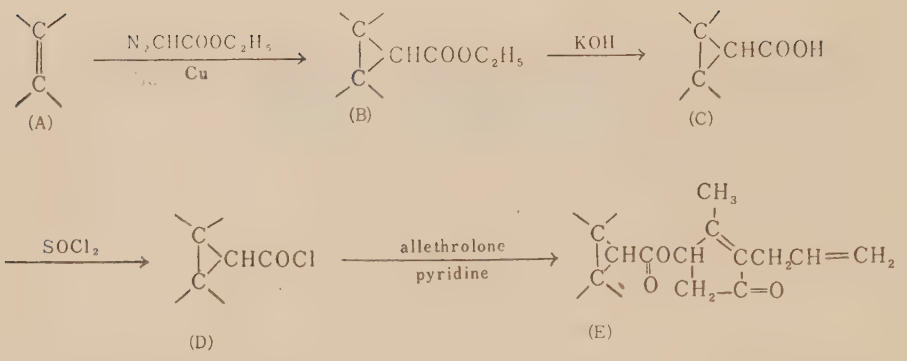
	R	R′
Pyrethrin	I, *cis*-CH_2-CH=CH-CH=CH_2	-CH_3
Pyrethrin	II, *cis*-CH_2-CH=CH-CH=CH_2	-$COOCH_3$
Cinerin	I, *cis*-CH_2-CH=CH-CH_3	-CH_3
Cinerin	II, *cis*-CH_2-CH=CH-CH_3	-$COOCH_3$
Allethrin	(*d*, *l*-), -CH_2-CH=CH_2	-CH_3

The elucidation of their structures and subsequent success in the syntheses thereof led, as a matter of course, to the preparation of many homologous "rethrins".

In search for a more active analogues, many attempts have been made on modifications in *cyclo*propanecarboxylic acid as well as *cyclo*pentenolone-moieties. It has been chiefly in the modification of the alcoholic part that successful results were obtained. Thus, allethrin has been introduced as an effective analogues which can compete with the natural pyrethrins in insecticidal activity and is now commercially produced for domestic uses.

On the other hand, the modifications in acid part of the rethrins had not been realized.

In the view mentioned above, the authors attempted to synthesize such *cyclo*propanecarboxylic acids as to give more effective esters with allethrolone. Some new allethronyl esters of *cyclo*propanecarboxylic acids were prepared and their insecticidal activities were tested against common housefly. Most of these esters showed little activities, but allethronyl esters of substituted-phenyl *cyclo*propanecarboxylic acids were more or less toxic. Thereupon, the authors synthesized such *cyclo*propanecarboxylic acids to get some substituted-phenyl groups attached to the *cyclo*propane ring, and tested their insecticidal activities to clarify the relationship between the toxicity and the substitution on the *cyclo*propane ring.



Many synthetic methods for *cyclo*propane ring have been known, in which the most common one consists in the addition of diazo-compounds to olefinic compounds. In order to obtain the *cyclo*propanecarboxylic acids, the authors added ethyl diazoacetate to unsuturated hydrocarbons (A) and ethyl *cyclo*propanecarboxylates (B) were subsequently saponified to the expected acids (C). Allethronyl esters of these acids were prepared by the usual method through the acid chlorides (D), and were purified by chromatography on alumina column. The insecticidal activities of these allethronyl esters (E) were tested against common housefly, *Musca domestica vicina* Macq.

The chemical structures of these acids and the results of bioassay are listed in Table I.

As is apparent from Table I, allethronyl 2-phenyl*cyclo*propane-1-carboxylate (III) and 2-methyl-3-(3′-methoxyphenyl)-*cyclo*propane-1-carboxylate (VI) which have no methylenedioxy group on the phenyl, showed a little insecticidal activities. Moreover allethronyl 2-piperonyl-*cyclo*propane-1-carboxylate (XII) was nontoxic, but allethronyl 2-methyl-3-(3′,4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate (XV) showed remarkable activities, and the relative effectiveness was calculated as 0.18 on mortality, and 0.40 on knock-down activity as compared with *α-dl-trans*-allethrin.

From these results, the authors concluded that the insecticidal activities of these allethronyl esters depend to some extent on the existence of 3,4-methylenedioxyphenyl group as one of the substituents on the *cyclo*propane ring, and attempted to synthesize such *cyclo*propanecarboxylic acids.

As the simplest *cyclo*propanecarboxylic acid containing 3,4-methylenedioxyphenyl group on the *cyclo*propane ring, 2-(3′,4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylic acid (VIII) was synthesized by the addition of ethyl diazoacetate to 1-vinyl-3,4-methlenedioxybenzene (VII)[1], and two isomeric acids were obtained by fractional crystallization (VIIIa, m.p. 144～5°, VIIIb, m.p. 121～2°). Both of their allethronyl esters (IXa, IXb) were less toxic than previously synthesized allethronyl 2-methyl-3-(3′, 4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate (XV). The fact that allethronyl 2-(3′,4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylates (IX) were less toxic than allethronyl 2-methyl-3-(3′, 4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate (XV), suggests that the insecticidal activities of such allethronyl esters may be affected by the existence of methyl group on the *cyclo*propane ring.

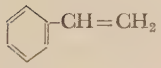
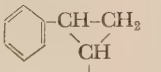
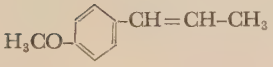
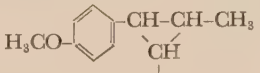
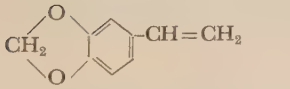
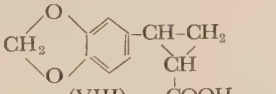
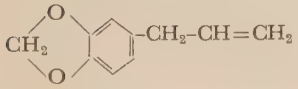
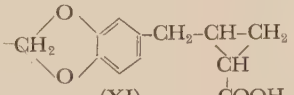
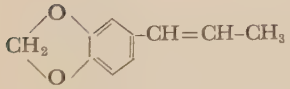
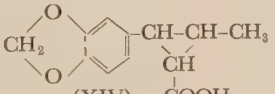
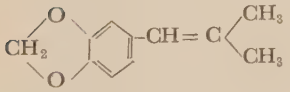
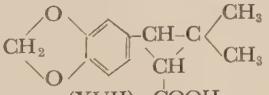
The suggestion mentioned above led that allethronyl 2,2-dimethyl-3-(3′,4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate might be more toxic. Piperonal was treated with isopropylmagnesiumbromide, and following dehydration of the resulting 1-methyl-2-(3′, 4′-methylenedioxyphenyl)-propanol, 1-(isobut-1′-enyl)-3,4-methylenedioxybenzene (XVI) (b. p. 94.5～95.5°/2 mm) was obtained. This aralkenyl

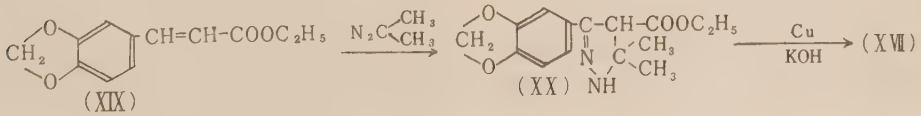
1) A. Klages, *Chem. Ber.*, **36**, 3595 (1903).

compound (XVI) was treated with ethyl diazoacetate in the usual way. After the saponification of the resulting ester, the acid (XVII) was fractionally crystallized to give two isomeric acids (XVIIa, m.p. 171~2°, XVIIb, m.p. 134~5°). The allethronyl esters of these acids (XVIIIa, XVIIIb) was the most toxic among allethronyl esters previously synthesized by the authors, and its relative toxicities were calculated as 1.48 times more active on the mortality and 1.21 times on the knock-down activity than *α-dl-trans*-allethrin as the standard.

Afterwards, the authors obtained the same acid (XVIIb) by the other route, i.e. the addition of dimethyldiazomethane to ethyl piperonylideneacetate (XIX) to yield a pyrazoline ester (XX) (m.p. 142~3°). This pyrazoline ester (XX) was submitted to thermal decomposition, and the subsequent hydrolyses of the resulting ester yielded an acid m.p. 134~5° exclusively. This acid was shown to be identical with previously synthesized acid (XVIIb) (m.p.

TABLE I.

Unsaturated compounds Structure & Code	*Cyclo*propane carboxylic acids Structure & Code	Allethronyl *cyclo*propanecarboxylates Code	Insecticidal activity: Knock-down % after 30 mins.	Insecticidal activity: Mortality after 24 hrs.
 (I)	 (II)	(III)	0.0	−
 (IV)	 (V)	(VI)	0.0	+
 (VII)	 (VIII)	(IX) a	67.9	+++
		(IX) b	0.0	+
 (X)	 (XI)	(XII)	0.0	+
 (XIII)	 (XIV)	(XV)	100.0	++++
 (XVI) *α-dl-trans*-allethrin	 (XVII)	(XVIII) a	100.0	++++
		(XVIII) b	100.0	++++++
			100.0	+++++



134~5°) by the other route using ethyl diazoacetate, by the mixed melting point comparison as well as by the complete identity of IR-spectra.

EXPERIMENTAL

The general procedure for the preparation of *cyclo*propanecarboxylic acids using ethyl diazoacetate and for the esterification with allethrolone, and the insecticidal tests were carried out as examplified in the following:

a) Preparation of *cyclo*propanecarboxylic acid. The unsaturated hydrocarbon (A) was heated to 110° in ligroin (b. p. 110~120°) and in the presence of copper powder (5 g/1 mole of A). To the mixture, ethyl diazoacetate (0.8~1 mole/1 mole of A) was added dropwise within 2 hrs. so as to keep the reaction temperature at 110~120°. After the evolution of nitrogen was over, the reaction mixture was cooled and copper powder was filtered off. From the filtrate, unreacted original compound (A) and ligroin were recovered. Since the resulting ethyl *cyclo*propanecarboxylate (B) has high boiling points in general, the residue of the evaporation was directly hydrolyzed by refluxing with 10% alcoholic KOH on a water bath. The acid (C) was isolated in the usual manners and was purified by fractional crystallizations.

b) Esterification with allethrolone. The acid (C) dissolved in chloroform containing thionylchloride (1.2 mole/1 mole of C), was set aside for 1 day or was refluxed on a water bath for 3 hrs.. After it, chloroform and the excess thionylchloride were completely removed in vacuo, the remaining acid chloride (D) was pure enough for further use.

The acid chloride (D) in dry benzene was added to the solution of allethrolone (0.8 mole/1 mole of C) in benzene and dry pyridine. The reaction mixture was kept at room temperature for 24 hrs., then the neutral product containing the ester (E) and unreacted allethrolone was extracted with ether. The extract was separated by elution chromatography using Al_2O_3 for the absorbant and eluted with petroleumether-ether (3 : 1). These esters were characterized by the absence of the OH band in the IR-spectra.

c) Bioassay. The common housefly, *Muscsa domestica vicaina* Macq., was reared with culture medium, and 3 or 4 days individuals after emergence were used. For the insecticidal test of the ester (E), two methods were employed. One of them is the settling mist apparatus method in 1% refined kerosene solution to examine the knock-down effect after 30 mins., and the other is the topical application method by microsyringe in 1% aceton solution to examine the mortality after 24 hrs.. The relative effectiveness of these esters were calculated from their median knock-down doses and median lethal doses.

2-Phenyl*cyclo*propane-1-carboxylic acid (II) m. p. 146~7°, Yield 37%, (*Anal.* Found: C, 74.13, H, 6.33; Calcd. for $C_{10}H_{10}O_2$: C, 74.05, H, 6.22).

Allethronyl 2-phenyl*cyclo*propane-1-carboxylate (III) n_D^{20} 1.5518.

2-Methyl-3-(4′-methoxyphenyl)-*cyclo*propane-1-carboxylic acid (V) m. p. 121~2°, Yield 24%, (*Anal.* Found: C, 69.78, H, 6.93; Calcd. for $C_{12}H_{14}O_3$: C, 69.88, H, 6.84).

Allethronyl 2-methyl-3-(4′-methoxyphenyl)-*cyclo*propane-1-carboxylate (VI) n_D^{18} 1.5455.

2-(3′, 4′-Methylenedioxyphenyl)-*cyclo*propane-1-carboxylic acid (VIII) An acid (VIIIa) of m. p. 144~5° which dissolved mere easily in methanol than in chloroform, and an acid (VIIIb) of m. p. 121~2° which dissolved more easily in chloroform, were obtained by fractional recrystrllization. (VIIIa) Yield 9.5%, (*Anal.* Found: C, 64.11, H, 4.81; Calcd. for $C_{11}H_{10}O_4$: C, 64.07, H, 4.89), and (VIIIb) Yield 9.5%, (*Anal.* Found: C, 64.32, H, 5.05; Calcd. for $C_{11}H_{10}O_4$: C, 64.07, H, 4.89).

Allethronyl 2-(3′, 4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate (IX) The acids (VIIIa, VIIIb) were esterified with allethrolone respectively. (IXa) n_D^{22} 1.5642, (IXb) n_D^{22} 1.5410.

2-Piperonyl*cyclo*propane-1-carboxylic acid (XI) m.p. 128~9°, Yield 5%, (*Anal.* Found: C, 65.30, H, 5.94; Calcd. for $C_{12}H_{12}O_4$: C, 65.44, H. 5.49).

Allethronyl 2-piperonyl*cyclo*propane-1-carboxylate (XII) n_D^{20} 1.5470.

2-Methyl-3-(3′, 4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylic acid (XIV) m. p. 134~5°, Yield 11%, (*Anal.* Found: C, 65.30, H, 5.66; Calcd. for $C_{12}H_{12}O_4$: C, 65.44, H, 5.49).

Allethronyl 2-methyl-3-(3′, 4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate (XV) n_D^{23} 1.5482.

2, 2-Dimethyl-3-(3′, 4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylic acid (XVII) a) Piperonal (30 g, 0.2 mole) in 200 ml of ether was added to the Grignard reagent which was prepared from magnesium (4.9 g, 0.2 mole) and isopropylbromide (28 g, 0.2 mole) at room temperature. After hydrolysis with cold 10% H_2SO_4, the organic layer was separated and the aqueous layer was extracted with ether. After the solvent was distilled off, the extract containing 1-(3′,4′-methylene-

dioxyphenyl)-2-methylpropanol was diluted with 200 ml of dry benzene and refluxed with P_2O_5 on a water bath for 3 hrs.. The reaction mixture was poured into water to decompose the excessive P_2O_5. 1-(Isobut-1′-enyl)-3,4-methylenedioxybenzene (XVI) was isolated in the usual way, b. p. 94.5~95.5°/2 mm, n_D^{20} 1.5605, Yield 32 g (91%).

The compound (XVI) was treated with ethyl diazoacetate following the general procedure, and the acid (XVII) was separated into two isomers by fractional recrystallization from methanol, (XVIIa) m. p. 170~1°, Yield 6.4%, (*Anal.* Found: C, 66.50, H, 5.90; Calcd. for $C_{13}H_{14}O_4$: C, 66.65, H, 6.20) and (XVIIb) m. p. 134~5°, Yield 19%, (*Anal.* Found: C, 66.37, H, 6.11; Calcd. for $C_{13}H_{14}O_4$: C, 66.65, H, 6.20).

b) Dimethyldiazomethane[2] (*ca.* 3 g, 0.14 mole) was added to ethyl piperonylideneacetate (XIX) (2.9 g, 0.14 mole) in chilled (−10~−20°) xylene solution and the reaction temperature was kept at −10~−20° for 3 hrs. and then at room temperature for 24 hrs.. After removal of xylene, a crystalline pyrazoline ester (XX), m. p. 142~3°, (*Anal.* Found: C, 62.02, H, 6.31, N, 9.65; Calcd. for $C_{15}H_{18}O_4N_2$: C, 62.05, H, 6.25, N, 9.65) was isolated.

The thermal decomposition of the pyrazoline ester (XX) in the presence of copper powder took place at an elevated temperature (170~190°) under evolution of nitrogen, and subsequent distillation in vacuo of the residue gave the expected ester, b. p. 130~1°/2 mm, n_D^{18} 1.5340, Yield 1.5 g, (13% based on dimethyldiazomethane). This ester was hydrolysed with 10% alcoholic KOH, and the acid of m. p. 134~5° was obtained. This acid was shown to be identical with the acid (XVIIb) previously synthesized by the other route using ethyl diazoacetate, by the mixed melting point comparison as well as by the complete identity of IR-spectra.

Allethronyl 2,2-dimethyl-3-(3′, 4′-methylenedioxyphenyl)-*cyclo*propane-1-carboxylate (XVIII) The acids (XVIIa, XVIIb) were esterified with allethrolone respectively. (XVIIIa) n_D^{20} 1.5410, (XVIIIb) n_D^{18} 1.5450.

2) S. Takei, T. Sugita and Y. Inouye, *Ann.*, **618**, 108 (1958).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 464~467, 1960]

Studies on the Protease of *Pseudomonas*

Part VI. On the Role of Ca in the Enzyme Production (supplemented)

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Received December 7, 1959

A discussion was made on the step of the incorporation of Ca in the synthesizing reaction of the proteinase of *Ps. myxogenes* sp.

When the cell suspensions containing a carbon source without addition of Ca were shaken aerobically, the secreted protein content was found to be remarkably lower than that of the suspensions containing Ca, while total amounts of secreted organic nitrogen materials were almost the same despite of the presence or absence of Ca. From the experimental result of an auto-splitting phenomenon of the proteinase by Ca-removal treatment, it was assumed that the above result occurs by auto-digestion of the proteinase secreted in the medium where Ca is absent. Accordingly, the step of incorporation of Ca would be considered to occur in the synthesized Ca-free proteinase.

INTRODUCTION

In a previous paper[1] of this series, it has been shown that Ca, which is required as an essential factor[2] for the proteinase production of *Ps. myxogenes* sp., is a constituent of the produced enzyme itself, while the removal of Ca from the enzyme molecule causes inactivation. Further, it has also been indicated[3] that the resting cells of the bacteria could produce the enzyme by shaking them with the addition of only a carbon source and Ca ion without accompanying growth. That is, nitrogen material within the cells contributes to the enzyme synthesis. Further, from the experiment on amino acid analogues[3] in the enzyme production, it was assumed that in any step of the enzyme-synthesizing reaction Ca might be required from the precursor substance which is a more complicated substance than amino acids.

Therefore, the following two pathways are to be considered for the problem concerning the step of incorporation of Ca in the enzyme-synthesizing reaction;

1. Precursor+Ca→Precursor-Ca→Proteinase
2. Precursor→Ca-free
   Proteinase+Ca→Proteinase

In order to clarify this problem, the following experiment was performed.

EXPERIMENTAL AND RESULTS

I. Researches for other substances constituted with Ca

As will be seen in the following paper[4], a precursor material immunologically related to the proteinase cannot be found within the cells. However, whenever a protein or high molecular peptide which incorporates with Ca within the cells at the time when the cells are shaken for enzyme formation, is formed this protein or peptide might be assumed to be the precursor of the enzyme. This also may clarify that the incorporation of Ca occurs at a precursor state of enzyme synthesis.

The following experiment was undertaken to clarify this question. The washed cells of the bacteria was prepared as already shown in the previous paper[3]. Each

1) K. Morihara, This Bulletin, **23**, 60 (1959).
2) K. Morihara, *ibid.*, **20**, 243 (1956).
3) K. Morihara, *ibid.*, **23**, 49 (1959).
4) K. Morihara, This Bulletin, **24**, 467 (1960).

100 ml contained 200 mg of the cells as dry weight in M/50 phosphate buffer of pH 7.0, with 1% fructose, and 10^{-3} M $CaCl_2$ composed of 20 μC $Ca^{45}Cl_2$ of final concentration, and was shaken aerobically in 500-ml shaking flasks at 30°C for 5, 10 and 16 hrs., respectively. Cells at various ages were collected by centrifugation, washed with distilled water several times, and crushed with alumina in the cold. After centrifugation at 10,000 r. p. m. for 30 minutes in the cold, the proteolytic inactive clear supernatant was subjected to paper-electrophoresis using M/20 Tris-buffer (pH 8.5) containing M/100 Ca-acetate as a buffer solution by 0.5 mA/cm at about 5°C. After a 16-hours run, the paper was dried in the atmosphere. The detection of protein or peptide was performed by Amidoschwarz, brom phenol blue or ninhydrin on a part of the paper, and contact printing to X-ray film (radio-autography) on the remaining strip was made for about two months. Exposion of X-ray film was not observed at all in the fraction corresponding to the colour reaction of protein or peptide.

From the result obtained, it can be said that the protein or peptide constituted with Ca is not formed within the cells at the time when enzyme production occurs.

II. Differences in characters of the organic nitrogen materials secreted from the cells in the presence or absence of Ca ion

In the previous paper[3], it has been shown that the maximum activity attained by cell suspensions with a carbon source is remarkably lowered when Ca ion is added after more than an 8-hours shaking period. The same phenomenon was also observed in the washed cells which were previously shaken with a carbon source even in the presence of Ca ion for more than 8-hours. On the other hand, decrease of the enzyme producing ability was not observed in those cells which were shaken for 24 hrs. without a carbon source or even in the presence of not only carbon but also nitrogen sources.

Accordingly, it could be considered that the nitrogen substance which should be transformed to the enzyme would be consumed by shaking cells with a carbon source despite of the presence or absence of Ca ion, that is, in both cases of production or no-production of the enzyme. Hence, the following question arises: what substance is secreted in the medium without Ca by consumption of nitrogen substance of cells, while the enzyme is seen at the extracellular site in the presence of Ca.

To clarify this question, the nature of the organic nitrogen materials secreted from the cells in either the presence or absence of Ca was examined.

Two hundred mg of the washed cells (as dry weight) which were prepared as shown in the previous paper[3] was added to the reaction mixture containing M/50 phosphate buffer of pH 7.0 in the presence or absence of 0.5% fructose and also 10^{-3} M $CaCl_2$, respectively, to make the total mixture up to 100 ml, in 500-ml shaking flasks, and incubated on a shaker at 30°C for 16 hours. Following this procedure, a clear supernatant was obtained from the reaction mixture by centrifugation at 5000 r. p. m. for 30 minutes.

The fructose content was measured by the method of Bertrand, and the proteolytic activity was determined by the usual method[2]. The contents of organic nitrogen materials were examined on 0.5 ml of the supernatant (made up to 6.5 ml, in final) at 500 mμ by the method of Lowry et al.[5]. The determination of the secreted protein content was made on one ml of the solution of the isolated materials from the supernatant, by the method of Lowry et al.[5] (final to 6.5 ml) at 500 mμ. Isolation was made from 50 ml of the supernatant by such treatments as rivanol precipitation (0.1%), elution with 0.5 M $CaCl_2$, fractional precipitation of acetone (40~60%) and the final volume was made up to 10 ml with water. The results are given in Table I.

From the table, it is clear that organic nitrogen substances are secreted in the medium by shaking the cells with fructose in almost the same amount regardless of the presence or absence of Ca, while only a little secretion is seen in the absence of carbon source. On the other hand, the content of protein secreted in the medium in the absence of Ca is found to be almost one-fifth of that observed in the presence of Ca. The above results show that a low molecular organic nitrogen material is secreted when the cells are shaken with a carbon source in the absence of Ca.

III. Effect of Ca-removal treatment on the nature of enzyme protein

The low molecular organic nitrogen material seen in the medium in the absence of Ca could be caused by autodigestion of the proteinase secreted in the medium. To clarify this point, the following experiment was performed.

A portion of 0.75 ml of the reaction mixture containing the crystalline proteinase (670 [u]/ml) and 0.25 M Tris-buffer of pH 8.0 was kept at 50°C for two hours in the presence or absence of 0.125 M EDTA. When treated in the absence of EDTA was proteolytic activity found to be unchanged (670 [u]/ml), while activity was remarkably diminished (19 [u]/ml) in the presence of

5) O.H. Lowry et al., *J. Biol. Chem.*, **193**, 265 (1951).

TABLE I. DIFFERENCES IN THE NATURE OF THE ORGANIC NITROGEN MATERIALS SECRETED BY THE CELLS IN THE PRESENCE OR ABSENCE OF Ca.

Composition of the reaction mixture		Fructose utilized mg/100ml	Proteolytic activity [u]/ml	Organic nitrogen contents secreted (OPT.D.)	Protein contents secreted (OPT.D.)
fructose	$CaCl_2$				
+	+	275	5.5	0.25	0.21
+	−	265	0.2	0.23	0.04
−	+	—	0.0	0.045	0.0

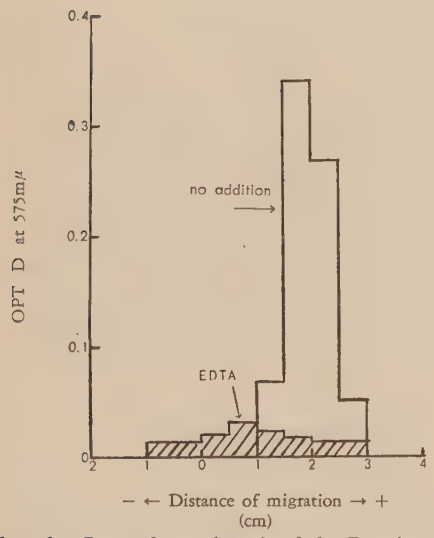

FIG. 1. Paper-electrophoresis of the Proteinase Treated in the Presence or Absence of EDTA at 50°C.

0.1 ml of the reaction mixture treated in the presence (▨) or absence (□) of 0.125 M EDTA at 50°C, was subjected to paper-electrophoresis. The phoresis was done by 0.5 mA/cm (450 V) at about 5°C for 16 hrs., using M/20 Tris buffer (pH 8.5) containing M/100 calcium-acetate as buffer solution.

EDTA.

To determine the protein nature of the enzyme inactivated by the treatment of EDTA, 0.1 ml of the treated reaction mixture was subjected to paper-electrophoresis (width of the paper 2 cm, length 15 cm). The buffer solution used was the same as that mentioned in section I of this paper, and phoresis was also done on the same condition. After drying in the atmosphere, the paper was immersed in 0.2% brom phenol blue solution saturated with $HgCl_2$, and then washed repeatedly by distilled water until the colour disappeared in the washed solution. The paper was cut into pieces, each 0.5 cm in length, eluted with 6.0 ml of 0.01 N NaOH, and the optical density read at 575 mμ within 30 minutes with a Beckmann photometer. The results are shown in Fig. 1.

The weak colour reaction of the inactivated enzyme solution treated with EDTA compared with that of its absence might be considered to be caused by splitting the enzyme protein into small fragments. Further, it was found that little precipitation is seen even if a two times volume of acetone is added to the inactivated enzyme solution.

Accordingly, it could be said that Ca-free proteinase cannot maintain its originl protein structure owing to instability occurring by auto-digestion or for other reasons.

SUMMARY AND DISCUSSION

The extracellular proteinase of *Ps. myxogenes* sp. is constitued with Ca as an essential component as shown in the previous paper[1]. This paper deals with a study concerning the step of incorporation of Ca in the synthesizing reaction of the proteinase of the bacteria.

A protein or peptide substance constituted from Ca is not found within the cells of the bacteria which have been shaken in the presence of a carbon source and Ca ion, while the proteinase is accumulated at the extracellular site. As it is sure that nitrogen material within the cells (which has been assumed to be a more complicated substance than amino acid, which is a precursor substance) contributes to the enzyme synthesis, the above result might suggest that Ca is not incorporated at the precursor state in the enzyme synthesis, or even if the incorporation really does occur at that state, the precursor substance is immediately secreted in the medium. However, we have not found the precursor material which is secreted in the medium where the material is activated autocatalytically as seen in *Streptococcal* proteinase[6].

6) S.D. Elliott, *J. Exptl. Med.*, **92**, 201 (1950).

Accordingly, it is difficult to consider the latter case to occur.

The total amount of organic nitrogen material secreted in the medium containing a carbon source by shaking the bacterial cells aerobically, is found to be identical in both the presence or absence of Ca ion, but the secreted protein content is remarkably diminished (to almost one-fifth) in the absence of Ca ion. Further, it is clear that the removal of Ca from the enzyme molecule, by treatment with EDTA at 50°C, results not only in inactivation of the enzymatic action but also exhibits the auto-splitting phenomenon of the enzyme protein.

These results may lead to the assumption that Ca-free proteinase would be secreted in the medium regardless of the existence of Ca ion, and the enzyme is accumulated when it is stabilized by the constitution of Ca ion in the medium, whereas the auto-splitting phenomenon of the enzyme would take place in absence of the Ca ion.

Therefore, the incorporation of Ca in the proteinase synthesis by the bacteria is considered to occur in the completely synthesized Ca-free proteinase secreted in the medium.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto University for his constant guidance and encouragement throughout this work. Thanks are also due to Mr. E. Masuo of this Laboratory for his assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 467~473, 1960]

Studies on the Protease of *Pseudomonas*

Part VII. An Immunological Study of the Crystalline Proteinase

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Received December 7, 1959

An immunological study was made on the crystalline proteinase of *Ps. myxogenes* sp.

By application of this method, it was shown that not only the determination of the purity of the enzyme which is used as an antigen, but a comparison of protein nature of the enzyme with proteinases of other origins is also attainable.

INTRODUCTION

Recently, in the study of enzymes, immunological methods have been applied to examine the protein nature[1~3] of enzyme and to analyze[4~7] the mechanism of enzyme formation.

Since the author has succeeded in the prepa-

1) S.D. Elliott, *J. Exptl. Med.*, **92**, 201 (1950).
2) T. Amano et al., *Med. J. Osaka Univ.*, **7**, 593 (1956).
3) T. Wada, *J. Biochem.*, **46**, 329 (1959).
4) M. Cohn and A.M. Torriani, *J. Immunol.*, **69**, 471 (1952).
5) M. Cohn and A.M. Torriani, *Biochim. Biophys. Acta*, **10**, 280 (1953).
6) M.R. Pollock, *J. Gen. Microbiol.*, **14**, 90 (1956); **15**, 154 (1956).
7) M. Nomura and T. Wada, *J. Biochem.*, **45**, 629 (1958).

ration of an antibody against the twice recrystallized proteinase[8] of *Ps. myxogenes* sp., an immunological study was attempted in order to examine the nature of this enzyme. It was found that determination of purity of the enzyme used as the antigen is carried out more accurately by employing immunological methods than by application of electrophoresis using Tieselius' apparatus. Further, it was shown that the proteinase of *Ps. myxogenes* sp. is comparable with the proteinases obtained from other origins.

The results obtained in this experiment are as follows.

EXPERIMENTAL METHODS

Twice recrystallized proteinase[8] of *Ps. myxogenes* sp. was used as the antigen. The crystals were dissolved in 0.85% NaCl solution at a concentration of 5 mg/ml and sterilized by filtration. The sterilized solution was injected intravenously into rabbits in increasing doses at the rate of one injection in every four or five days. The immunizing schedule was as follows: two injections of 0.1 ml, two injections of 0.3 ml, two injections of 0.5 ml, two injections of 0.8 ml. Allowing 7 days to elapse after the last injection, the rabbits were bled and the sera inactivated at 56°C for 30 minutes, added merzonine at a concentration of 0.01 per cent, and stored in a refrigerator.

The precipitation reaction between the antigen and its antibody was measured by the ring-test method using a small test tube.

The cells of *Ps. myxogenes* sp. were prepared according to the method previously described[9] and were washed with saline solution. The proteolytic activity was measured as usual[10].

Determination of electrophoretic pattern of active or inactive proteinase was performed by the following method. After drying the paper (width, 2 cm; length, 15 cm) which had been subjected to electrophoresis, it was immersed in 0.2% brom phenol blue solution saturated with $HgCl_2$ for 10 minutes, and then washed by distilled water repeatedly until the colour could not be detected in the washed solution. The treated paper was cut into pieces, each 0.5 cm in length, eluted with 6 ml of 0.01 N NaOH, and the optical density read at 575 mμ within 30 minutes.

Purified proteinase of various bacterial origins were prepared as follows: after shaking culture of the organism in Medium I (2% meat extract, 1% peptone, 0.8% glucose, M/150 $CaCl_2$, M/300 phosphate: pH 7.0) or in Medium II (2% glucose, 1% $(NH_4)_2HPO_4$, 1% $Na_2HPO_4 \cdot 12H_2O$, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract, 0.2% $CaCO_3$: pH 7.0) at 30°C for one or two days, a clear supernatant was obtained by centrifugation, from which the proteinase was purified by such procedures as salting out, dialysis, and fractional precipitation by acetone in the cold.

MATERIALS

Crystalline trypsin (Mochida Co.,) and purified pepsin (Wako Co., 1 : 10000) were used in this experiment. Crystalline proteinase of *B. subtilis* was obtained through the courtesy of Nagase Co., crystalline proteinase of *St. griseus* from Dr. Nomoto, purified proteinase of *Asp. saitoi* (purity, about 70%) from Dr. Yoshida and also various bacterial culture strains from Institute for Fermentation (Osaka) and Dept. of Science, Osaka University.

RESULTS

I. Immunological properties of the antiserum

Precipitation reaction between the crystalline proteinase of *Ps. myxogenes* sp. (*Ps* Enz) used as the antigen and its antiserum was measured. Antigen dilutions were performed, and the minimal concentration of *Ps* Enz which showed a clear white ring against the serum was found to be about 0.8 γ/ml.

Further investigation as to whether the serum reacts with the non-proteolytic materials stored within the cells was subsequently carried out. The wet cells of *Ps. myxogenes* sp. were crushed in alumina for about 30 minutes in the cold (0~5°C). The clear cell extract was prepared by the addition of saline solution in an adequate amount and centrifugation at 10,000 r.p.m. for 30 minutes.

The precipitation reaction between the clear extract and the antiserum was measured. Reaction was not observed until the extract reached a concentration of about 30~50 γ/ml, at which determination of the protein content was made by the method of Lowry et al.[11], using twice recrystallized proteinase of *Ps. myxogenes* sp. as

8) K. Morihara, This Bulletin, **21**, 11 (1957).
9) K. Morihara, *ibid.*, **23**, 49 (1959).
10) K. Morihara, *ibid.*, **20**, 243 (1956).
11) O.H. Lowry et al., *J. Biol. Chem.*, **193**, 265 (1951).

Table I. Precipitation Reaction by the Cross-absorbed Antiserum

Antiserum	Precipitation reaction (γ/ml)								
	Ps Enz			boiled cell-extract			ground cell-extract		
	30	6	1.2	40	8	1.6	150	100	50
No treatment	+	+	+	+	+	+	+	+	+
Ps Enz absorbed	−	−	−	+	+	+	+	+	+
boiled cell-extract absorbed	+	+	+	−	−	−	−	−	−

Symbols: + shows the reaction as positive, and − as negative.

the standard.

Further study revealed that the boiled extract of the cells—boiling a concentration of 50 mg cells (dry weight) per ml for one hour and centrifuged—showed a strong reaction on the antiserum almost equal to that of the *Ps* Enz mentioned above.

It would be very interesting to analyze the mechanism of the enzyme formation, if the reaction between the cell-extract and the antiserum is caused by the same antigenic components as *Ps* Enz. Thence the following cross-absorption experiment was carried out.

The *Ps* Enz absorbed, or the boiled cell-extract absorbed antisera, were prepared by the following procedures: to 0.9 ml of antisera 0.3 mg antigen proteinase in 0.1 ml saline or 0.4 mg of the boiled cell-extract in 0.1 ml saline was added, left overnight in a refrigerator, and the precipitate removed by centrifugation at 10,000 r. p. m. for 30 minutes in the cold. Precipitation reaction between the various absorbed antisera with *Ps* Enz, boiled cell-extract and ground cell-extract was measured, with results as shown in Table I.

From the above table it will be seen that absorption of the antiserum with *Ps* Enz removed all precipitins from the extract without significantly altering the capacity of serum to react with the cell-extract materials, while absorption with the boiled cell-extract did not affect the precipitin titer for *Ps* Enz but only affected the cell-extracts in both treatments.

From these results, it could be said that the precipitation reaction of non-enzymatic material stored within the cells occurs not by cross-reaction, and can be ascribed to inhomogeneity of the antigen proteinase.

II. Effect of inactivation of *Ps* Enz on its immunological behavior

It has already been shown[8),12)] that the proteolytic activity of *Ps* Enz is inactivated by heat- or acid-treatment, or by addition of EDTA at 50°C. To find the relation between enzymatic activity and immunological behavior, the precipitin titer of the inactivated enzyme solution was measured, and the results are shown in Table II.

As seen it is clear that inactivation of the enzymatic activity results in a parallel decrease of the precipitin titer. As the precipitin titer of the boiled cell extract was shown not to be affected by such treatments mentioned in the table, it could be said that a remarkable decrease of the precipitin titer was occurred by inactivation of the proteinase itself, and not by the impure

Table II. Inactivation of the Crystalline Proteinase and its Immunological Behavior

A portion of 0.75 ml of the reaction mixture containing the crystalline proteinase (670[u]/ml) and 0.25M Tris-buffer of pH 8.0 was kept at various conditions as illustrated in the table.

Treatment	Proteolytic activity		Precipitation reaction	
	[u]/ml	ratio of inactivation	Titer*	ratio of inactivation
		%		%
no treatment	670	—	×1600	—
63°C, 2 hrs.	31	95	× 100	94
100°C, 30 mins.	2.7	99.6	× 100	94
0.125 M EDTA (in final concn.), 2 hrs.	19	97.1	× 50	97
pH 3, 30 mins.	294	56	× 800	50

* Final dilution of the antigen which shows a clear white ring against the antiserum.

12) K. Morihara, This Bulletin, **23**, 60 (1959).

material attached to the preparation. This fact might lead the author to the next consideration that purity of the crystalline proteinase used as antigen exceeds a concentration of 94 per cent.

Even when a two times volume of acetone was added to the inactivated enzyme solution treated as seen in Table II, such precipitation as seen in the active proteinase was hardly observed. Subsequently, each 0.1 ml of the inactivated enzyme solutions was subjected to paper-electrophoresis, and the colour reaction by brom phenol blue was measured as described in the section "Method". Fig. 1 gives the results excluding the result of EDTA-treatment which has been shown in the previous paper[13]. The protein component of the enzyme inactivated may be seen to not only be dispersed but also remarkably weakened in its colour reaction to brom phenol blue. This might show that the inactivation results in auto-splitting of the enzyme protein, and accordingly, it would be natural to observe a paralleled decrease of the precipitin titer by inactivation of the enzyme.

III. Inhibition of proteolytic activity of *Ps* Enz by its antibody

Many workers[2,3,7,14,15] have demonstrated the inhibition of enzymatic activity by its homologous antibody. The author has here examined the inhibition of the proteolytic activity of *Ps* Enz by its antiserum. As seen in Fig. 2, a linear relationship was obtained between the degree of inhibition and quantity of the antiserum used here, and complete inhibition of the enzymatic activity was attained.

IV. The reaction of the anti-*Ps* Enz-serum on various proteinase preparations of other origins

The following experiment was made to find whether the antiserum of *Ps* Enz could react with various proteinases of other origins.

Table III shows the results of the various samples of proteinase. Crystalline or purified proteinase preparations of various origins men-

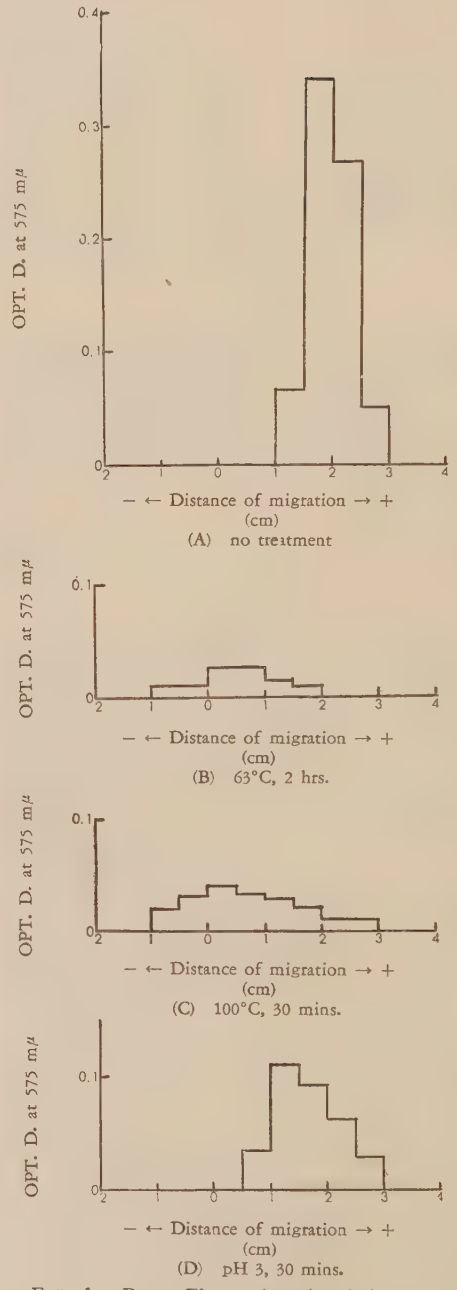

FIG. 1. Paper Electrophoresis of the Proteinase Inactivated by Various Treatments.

0.1 ml of the reaction mixture treated as in Table II, using M/20 Tris-buffer (pH 8.5) containing M/100 calcium-acetate as buffer solution was subjected to paper-electrophoresis. The phoresis was done by 0.5 mA/cm (450 V) at about 5°C for 16 hrs.

13) K. Morihara, This Bulletin, **24**, 464 (1960).
14) H. Lüers and F. Albrecht, *Fermentforschung*, **8**, 52 (1926).
15) H. Maeno and S. Mitsuhashi, *Symposia on Enz. Chem. (Japan)*, **13**, 215 (1958).

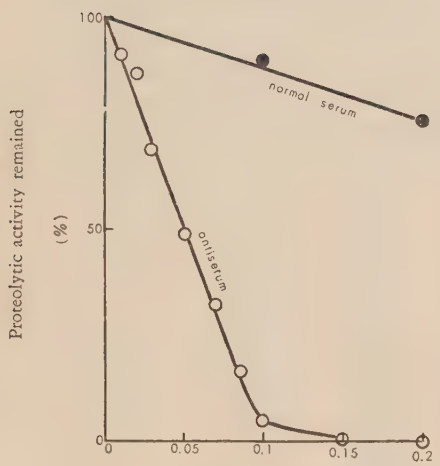

FIG. 2. Inhibition Curve of the Proteinase by its Antiserum.

0.4 ml of the crystalline proteinase solution (15 γ/ml) was mixed with normal-serum and its specific antiserum (volume is as seen in the figure) made up to 3.0 ml with 0.85% NaCl and after 60 minutes the remaining proteolytic activity was measured.
normal-serum ●—●
antiserum ○—○

TABLE III. REACTION OF THE ANTI-*Ps* ENZ-SERUM WITH SOME OTHER PROTEINASES

Proteinase*	Final concentration showing the precipitation reaction (/ml)
crystalline trypsin	2 mg
crystalline proteinase of *B. subtilis*	〃
crystalline proteinase of *St. griseus*	〃
purified pepsin (1 : 10,000)	2.5 mg
purified proteinase of *Asp. saitoi* (purity 70%)	2 mg

* The source of the various proteinases is shown in the "Method" of this paper.

tioned in the table cannot react with the antiserum until a concentration of over 2 mg/ml is attained, while *Ps* Enz reacts at a concentration of about 0.8 γ/ml. That is to say, all the samples tested are not related with *Ps* Enz in their immunological behavior.

Next, the immunological behavior of purified proteinase from various bacterial origins differing in genus or species was examined using ring-test method and neutralizing power of the antiserum, and the results are shown in Table IV.

As seen in the table, proteinases obtained from two strains of *Ps. aeruginosa* are shown to react on the anti-*Ps* Enz-serum. While, proteinases from other *Pseudomonas* such as *Ps. fluorescens*, *Ps. chlororaphis* and *Ps. riboflavinus*, or from the other genus such as *B. subtilis*, *B. circulans*, *B. vulgatus*, *Se. marcescens* FH, *M. citraus* and *M. flavus* do not show any positive reaction. Among various strains of *Pseudomonas* species used in this experiment, IFO 3383 *Ps. malvacearum*, IFO 3458 *Ps. fragi*, IFO 3508 *Ps. tabaci*, IFO 3460 *Ps. graveolens*, IFO 3310 *Ps. coronafaciens*, and IFO 3309 *Ps. striafaciens* were found to be little producer of the proteinase either in Medium I or II.

From the above results, it is concluded that the proteinases of the two strains of *Ps. aeruginosa* mentioned above are related immunologically with the proteinase of *Ps. myxogenes* sp. which the authors identified[16] while all the other proteinases are not related. It is of interest to note that both strains of *Ps. aeruginosa* mentioned above cannot produce the proteinase in Medium I but they can produce it in Medium II as similarly as seen in *Ps. myxogenes* sp.[10].

DISCUSSION

The result of cross-absorption experiment shows that the proteinase secreted in the medium by *Ps. myxogenes* sp. is not immunologically related to the materials existig within the cells. Accordingly, it could be said that a precursor material of the enzyme, which has been assumed to be kept within the cells as shown in the previous paper[9], is different from the enzyme in its immunological behavior.

Amano et al.[2] have pointed out that in determination by the immunological method four times recrystallized Taka-amylase A preparation is still of inhomogeneous nature. Also, in the application of such method as cross-absorption, the presence of impurity—its percentage was assumed to be within 6 per cent as is seen in this paper—was found to be

16) H. Katagiri, N. Mugibayashi and K. Morihara, *Inst. Chem. Res. Kyoto Univ.*, **18**, 41 (1949).

TABLE IV. EFFECT OF THE ANTI-*Ps* ENZ-SERUM ON THE VARIOUS BACTERIAL PROTEINASES

Ring test was made as usual using the purified proteinase solution (the enzymatic activity of about 7~8 [u]/ml) containing 0.85% NaCl prepared as shown in the section "Method" of this paper.

Inhibition test was conducted in the following way. A portion of 0.4 ml of the purified proteinase solution as mentioned above (the enzymatic activity of about 7~8 [u]/ml) was mixed with 0.1 ml of normal or specific antiserum, made up with 0.85% NaCl to 3 ml and after 60 minutes, remaining proteinase activity was measured.

Strains*	Medium**	Ring test***	Proteolytic activity, [u]/ml			% of inactivation
			blank	normal-serum	antiserum	
IFO 3061 *B. subtilis*	I	−	1.1	1.1	1.1	0
IFO 3342 *B. circulans*	//	−	0.9	0.9	0.9	0
IFO 3037 *B. vulgatus*	//	−	1.1	0.7	0.8	0
1008 *Se. marcescens* FH	//	−	1.0	1.0	1.0	0
IFO 3332 *M. citraus*	//	−	1.0	0.8	0.8	0
IFO 3242 *M. flavus*	//	−	1.0	0.6	0.9	0
IFO 3080 *Ps. aeruginosa*	II	+	1.2	1.1	0.35	68
T. 30 *Ps. aeruginosa*	//	+	0.7	0.6	0.3	50
IFO 3081 *Ps. fluorescens*	I, II	−	1.0	0.9	1.0	0
IFO 3506 *Ps. chlororaphis*	I	−	0.9	0.8	0.9	0
IFO 3140 *Ps. riboflavinus*	//	−	0.6	0.6	0.6	0

* The strains of IFO and T 30 were supplied from the Institute for Fermentation (Osaka), and strain 1008 was from Fac. Sci. of Osaka Univ.

** The composition is referred to in the section "Method" of this paper.

*** + shows the reaction as positive, and − as negative.

attached in the twice recrystallized proteinase preparation of *Ps. myxogenes* sp., which had been ascertained[8] to be of homogeneous nature on electrophoresis using Tieselius' apparatus.

The proteinase of *Ps. myxogenes* sp. is shown to be immunologically related with the proteinase obtained from *Ps. aeruginosa*, while the other proteinases obtained from the bacteria of different species and genus such as *Ps. fluorescens*, *Ps. riboflavinus*, *Ps. chlororaphis*, *B. subtilis*, *B. circulans*, *B. vulgatus*, *Se. marcescens* FH. *M. citraus*, *M. flavus* and *St. griseus*, and also various proteinases of mould and animal origins are not related. According to the Bergey's mannual of Determinative Bacteriology (7th edition, 1957), the optimum temperature for growth of *Ps. myxogenes* is at about 22°C and that of *Ps. aeruginosa* is about 37°C. It has been shown[16] that the optimum temperature for growth of *Ps. myxogenes* sp., which has been used in the work of this series, is about 37°C. From the results of the immunological study and also the optimum temperature for growth, it might be considered that the bacteria identified as *Ps. myxogenes* sp. is belonged to the species of *Ps. aeruginosa*. This problem will be dissolved more clearly when the author has the chance of obtaining a type species of *Ps. myxogenes* in the near future.

Wada[3] has found that anti-Taka-amylase-antibody reacts specifically with the amylase of *Aspergillus* species such as *Asp. sojae*, *Asp. oryzae*, *Asp. awamori*, and *Asp. usamii* but not with those from the other genus, and demonstrated that immunological specificity of mould amylase may provide a clue for the classification of microorganisms. Our experimental result concerning the bacterial proteinase may support her view more accurately, for immunological specificities of the proteinases of various *Pseudomonas* species are shown to be different with each other.

SUMMARY

1) Antiserum against the proteinase of *Ps. myxogenes* sp. was produced in rabbits by injection of the antigen proteinase.

2) By cross-absorption experiments, the crys-

talline proteinase used as the antigen was found to be of inhomogeneous nature even when it was recrystallized twice.

3) Inactivation of the enzyme by various treatments, such as heat, acid and EDTA, markably caused the same effect in decreasing its precipitin titer.

4) The proteolytic activity of the enzyme was found to be inhibited by its antibody.

5) The effect of the antiserum on various proteinases of other origins was studied. It was found that the antiserum inhibits the proteolytic activities of both strains of *Ps. aeruginosa;* while the proteinases of the other bacterial origins of different species and genus, and also those of mould and animal origins are never affected by the antiserum.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. for his constant guidance and encouragement throughout this work. He is also indebted to Mr. S. Mayama of this Laboratory for his cooperation in carrying out this experiment. Thanks are also expressed to Dr. Nomoto of Scientific Research Institute, Dr. Yoshida of Noda Institute for Scientific Research, Nagase Ltd., Institute for Fermentation (Osaka) and Dept. of Science of Osaka Univ. for their kindly supplying various proteinase preparations and various different bacterial strains used here.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 473~480, 1960]

β-Phenylmercapto-*cis*- and *trans*-cinnamic Acids

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Received December 8, 1959

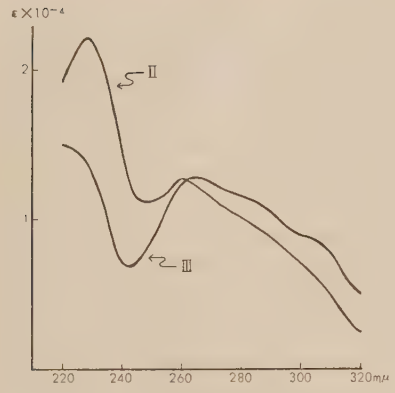
β-(*p*-Chlorophenyl) mercaptocinnamic acid and β-(*p*-acetamidophenyl) mercaptocinnamic acid were synthesized and separated into *cis*- and *trans*-isomers of each. Their configurations were determined by ring closure to be thioflavons under mild conditions. *trans*-Acids have an absorption band at 8.3μ while *cis*-acids do not have such band. Other physical methods generally used to determine the geometrical configuration were of no avail for these compounds carrying a bulky group at β-position. *cis*-Acid was produced via isomerization from the *trans*-ester during the procedure of saponification.

Some substituted β-phenylmercaptocinnamic acids have been prepared by hydrolysis of their esters which were obtained by addition of the corresponding sodium thiophenolates to esters of phenylpropiolic acid[1]. Each of the products was suggested to be a mixture of *cis*- and *trans*-isomers, since its melting point was not sharp. However, so far the separation of these isomers has not been resulted in a success. The case was found to be similar with the substituted β-phenoxycinnamic acids[2].

In the present preparation of β-(*p*-chlorophenyl) mercaptocinnamic acid by the analogous method, two kinds of crystals (II, III) were separated by the solubility of their alkali salts: the salt of III was readily soluble and the salt of II was sparingly

1) S. Ruhemann, *Ber.*, **46**, 3384 (1913).

2) S. Ruhemann and F. Beddow, *J. Chem. Soc.*, **77**, 984 (1900).



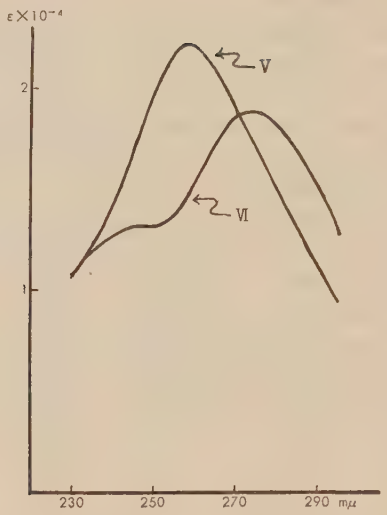
Wave length

FIG. 1. Ultraviolet Absorption Spectra of β-(*p*-Chlorophenyl) mercaptocinnamic Acids.
II: *cis*-Acid. III: *trans*-Acid.

soluble in water. It was shown by elemental analyses and a mixed melting point determination, that the free acids (II and III) were different substances of the same constitution. Reaction with phosphor pentachloride followed by Friedel Craft's reaction, gave the same 6-chlorothioflavon (VII) in a good yield, presumably involving isomerization of one isomer to the other as will be explained later. Thus II and III were supposed to be the geometrical isomers of β-(*p*-chlorophenyl) mercaptocinnamic acid.

By examination of ultraviolet absorption spectra of isomeric cinnamic acid, it has been recognized that the absorption band of "full-chromophore" shifts to a longer wave length and is more intensive in a *trans*-isomer than in the corresponding *cis*-isomer[3]. On the other hand, acidity of a *trans*-acid is, as generally accepted, usually weaker than the corresponding *cis*-acid by about 0.5 pKa unit[4].

However, the configurations of II and III could not be determined from these measurements (Table): III was absorbed at a longer wave length than II (Fig. 1), but the former was found



Wave length

FIG. 2. Ultraviolet Absorption Spectra of β-(*p*-Acetamidophenyl) mercaptocinnamic Acids.
V: *cis*-Acid. VI: *trans*-Acid.

to be stronger in acidity than the latter to be extent of 0.5 pKa unit.

When concentrated sulfuric acid was used as a reagent of ring closure, III gave thioflavon (VII) but II gave another product. Thus, III was supposed to be *trans*-acid.

β-(*p*-Acetamidophenyl) mercaptocinnamic acid prepared by the analogous procedure was fractionally recrystallized from alcohol into a more soluble product (V) and a less soluble one (VI). Their ultraviolet absorption curves are shown in Fig. 2. The shift of a band of absorption to a longer wave length is found in the curve of VI rather than in V, but extinction is more intensive in V than in VI. After the action of phosphor pentachloride followed by treatment with aluminium chloride at room temperature, both of them gave 6-acetamido-thioflavon (VIII). However, by employing the same treatment expect cooling the mixture in ice, the thioflavon (VIII) was produced from VI, but the starting material was almost quantitatively recovered from V. Thus, VI was decided to be *trans*-acid.

Infrared absorption spectra of these isomers are

3) E.A. Braude and E.S. Weight, in W. Klyne (eds.), Progress in Stereochemistry, **1**, p. 129 (1954), Butherworths Scientific Publications, London.

4) H.C. Brown, D.H. McDaniel and O. Häfliger, in E.A. Braude and F.C. Nachod (eds.), Determination of Organic Structure by Physical Methods, p. 583 (1955), Academic Press Inc., N.Y.

shown in Figs. 3 and 4. β-(*p*-Acetamidophenyl) mercapto-*trans*-cinnamic acid (VI) has a strong absorption at 8.3 μ but *cis*-acid (V) does not have such absorption band. These facts are also proved for β-(*p*-chlorophenyl) mercaptocinnamic acids. The ester of β-(*p*-acetamidophenyl) mercaptocinnamic acid, which has *trans*-configuration as mentioned below, has no absorption in this region (Fig. 5), and therefore this band seems to be due to the free carboxyl radical.

As a general rule, a *trans*-isomer has a higher melting point, lower solubility, an absorption band at a longer wave length and of higher intensity, and if an acid, weaker acidity than a *cis*-isomer. To determine the configurations of β-phenylmercaptocinnamic acids, these gener-

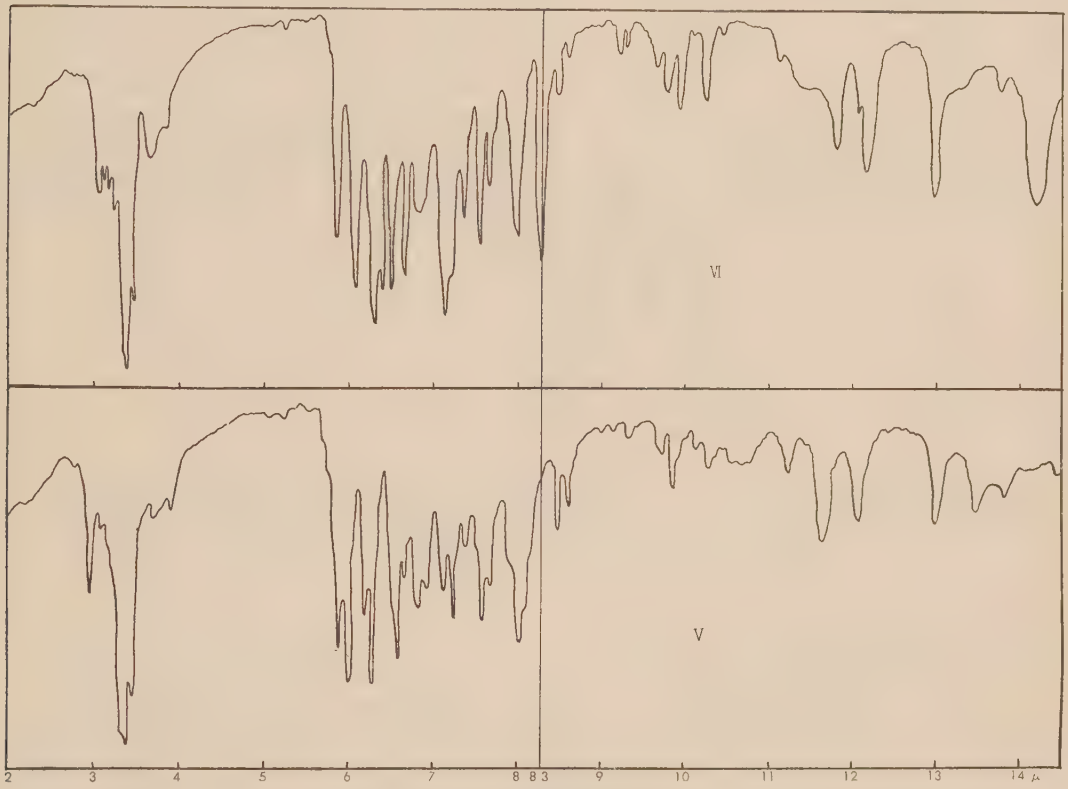


FIG. 3. Infrared Absorption Spectra of β-(*p*-Acetamidophenyl) mercaptocinnamic Acids.
V: *cis*-Acid. VI: *trans*-Acid.

PHYSICAL PROPERTIES OF β-PHENYLMERCAPTOCINNAMIC ACIDS.

p-$RC_6H_4SC(C_6H_5)=CHCOOH$

	R	Configuration	m.p.	Solubility	λ_{max} mμ	ε	ΔpKa *trans-cis*	IR band in 8.3μ
II	Cl	*cis*	174~6°	(salt/water)	260	12,700		−
					228	22,200		
III	Cl	*trans*	172~3°	∧	265	12,800	−0.15	+
V	CH_3CONH	*cis*	226°	(acid/alc.)	257	22,100		−
VI	CH_3CONH	*trans*	201°	∨	274	18,800		+

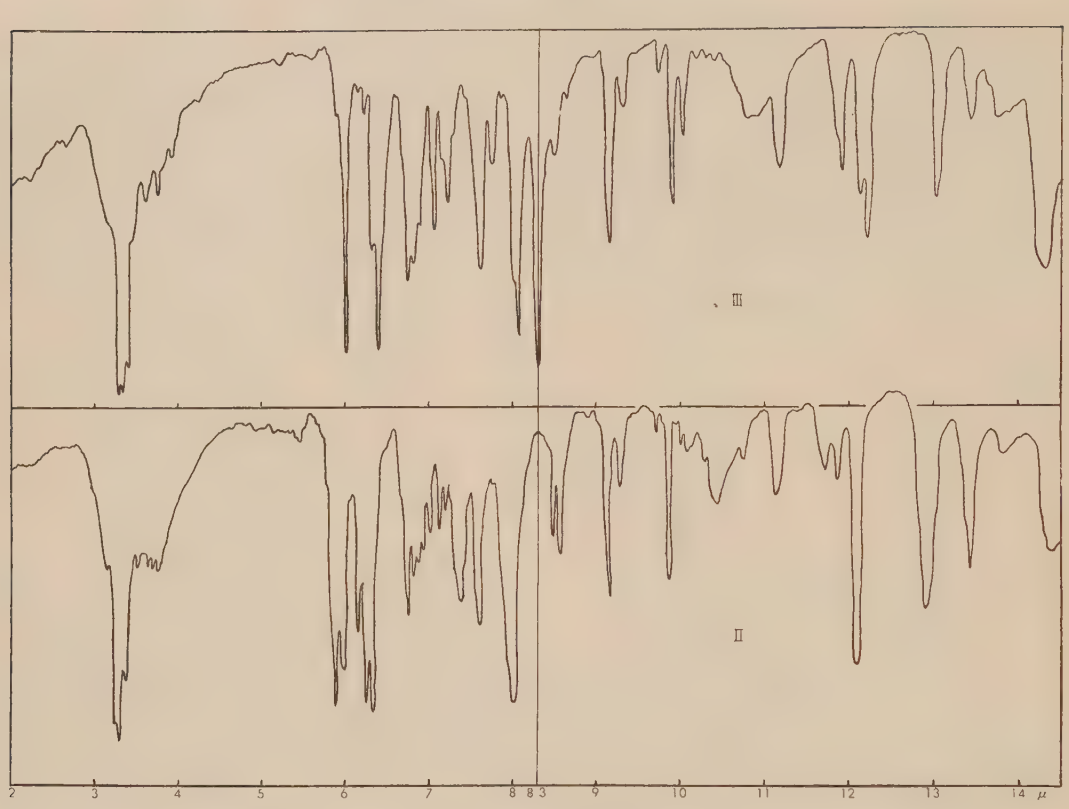


FIG. 4. Infrared Absorption Spectra of β-(*p*-Chlorophenyl) mercaptocinnamic Acids.
II: *cis*-Acid. III: *trans*-Acid.

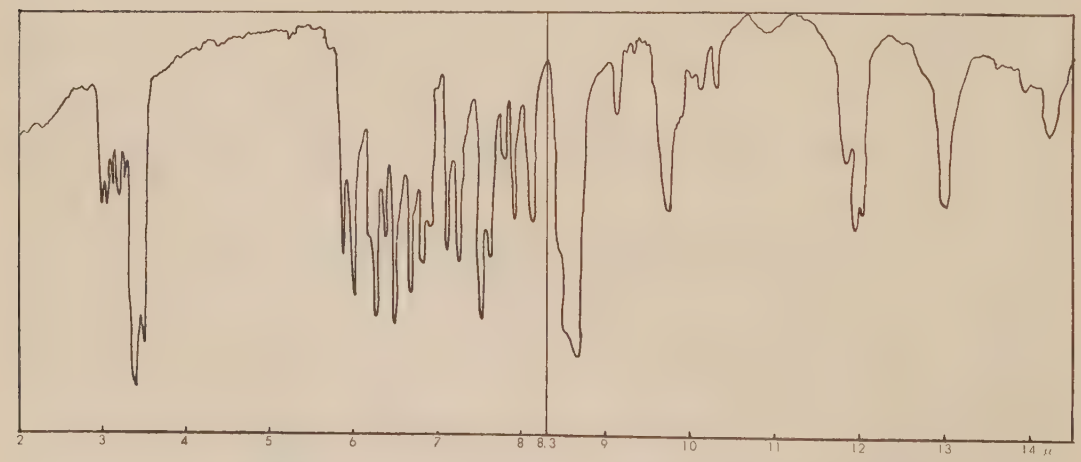


FIG. 5. Infrared Absorption Spectra of Methyl β-(*p*-Acetamidophenyl) mercaptocinnamate (IV).

alities as shown by their physical properties, are of no avail (Table). The alteration of generality may be due to the steric effect of a large group at the β-position.

Truce and his collaborators concluded that nucleophylic addition to triple bond proceeds in *trans*-sense[5] with one exception[6]. Ruhemann et al.[7] found that additional reaction of sodium phenolate to the ester of phenylpropiolic acid gives only one isomer at first, though it afterwards isomerizes into the other isomer for long standing. Also, in the present study where *p*-acetamidothiophenol was added to methyl phenylpropiolate (IX) to give methyl β-(*p*-acetamidophenyl) mercaptocinnamate (IV), only one isomer was obtained as a solid substance. This isomer was presumably assigned as *trans* by analogy with Truce's finding.

The product obtained from the purified adduct

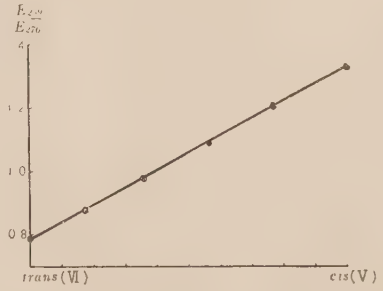
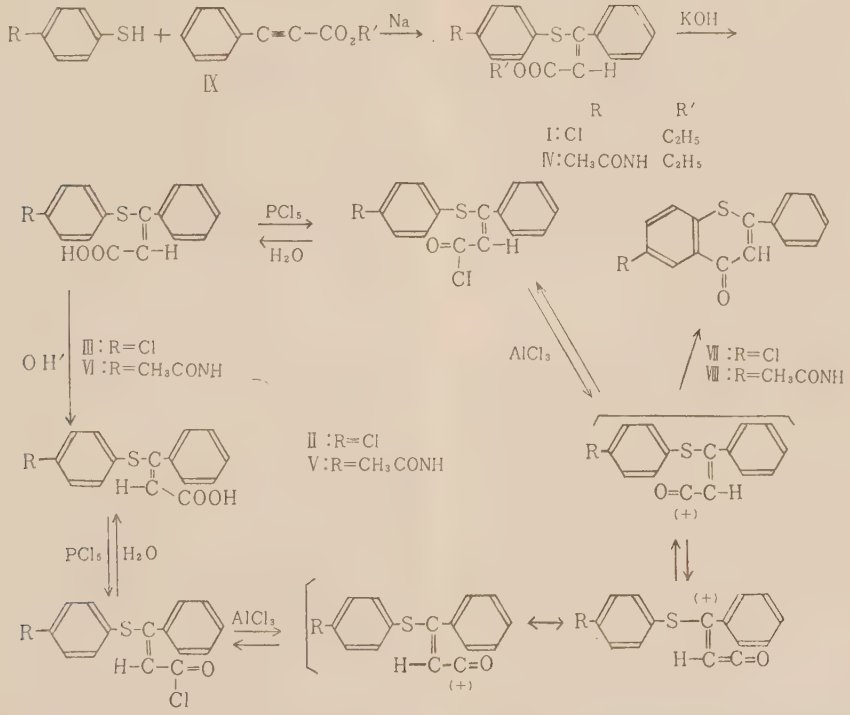


FIG. 6. The Relationship between E_{259}/E_{276} and Composition of Isomers of β-(*p*-Acetamidophenyl)-mercaptocinnamic Acids.

CHART



5) W.E. Truce, J.A. Simms and M.M. Boudakian, *J. Am. Chem. Soc.*, **78**, 695 (1956). W.E. Truce and J.A. Simms, *J. Am. Chem. Soc.*, **78**, 2756 (1956).

6) W.E. Truce and R.F. Heine, *J. Am. Chem. Soc.*, **79**, 5311 (1957).

7) S. Ruhemann and F. Beddow, *J. Chem. Soc.*, **77**, 1119 (1900).

ester (IV) by refluxion in alcohol (95%) containing approximately one equivalent of potassium hydroxide for three and a half hours was determined by ultraviolet absorption spectrometry and found to be a mixture of *cis* (V)- and

trans (VI)-isomers of corresponding acid. Spectrometrical determination of the mixture is based upon the fact that there is a linear relationship between the ratio of absoption at 259 mμ to that at 276 mμ and the composition of isomers (Fig. 6). The ratio of absorption of the saponified product was 0.84. This showed that the saponified product consisted of 90 percent *trans*-acid and 10 percent *cis*-acid. When *trans*-acid (VI) was treated in a similar way, the ratio changed from 0.79 to 0.84. This showed that the *trans*-acid is converted to *cis*-acid (V) giving a mixture, the composition of which was the same as the saponified product. An extremely rational explanation of these facts can be made by assuming that the adduct ester has a *trans*-configuration and *cis*-acid is produced via isomerization in the procedure of saponification of the ester.

By the action of alminium chloride on the chlorides of the acids, isomerization took place readily as well as the ring closure reaction into thioflavon. Thus, when *cis*-isomer (V) was used as the starting material, *trans*-isomer (VI) was obtained besides the thioflavon (VIII). When *trans*-isomer (VI) was the starting material, a considerable amount of *cis*-isomer (V) was found besides VIII (see Chart).

EXPERIMENTAL[8]

Ethyl β-(*p*-chlorophenyl) mercaptocinnnamate (I) was prepared according to the procedure of Ruhemann[1]. A mixture of 6 g of *p*-chlorothiophenol and 0.8 g of powdered sodium in dry xylene was refluxed until metallic sodium disappeared. To the magma was added 6 g of ethyl phennylpropiolate (IX) at 120°. After the mixture was successively washed with dilute sulfuric acid, dilute caustic alkali, and water, the xylene layer was dried with anhydrous sodium sulfate and evaporated, and the remaining oil was distilled in vacuo: b. p. 162° (10^{-2} mm), yield 5 g.

β-(*p*-Chlorophenyl) mercapto-*cis*-and *trans*-cinnamic acid (II, III). A mixture of 11 g of I and 2.7 g of potassium hydroxide in alcohol was refluxed for two hours. This produced crystals which were separated from the mother liquid by filtration. The crystals were dissolved in warm water and precipitated by the addition of dilute hydrochloric acid. The precipitate was filtered, washed with cold water, and then recrystallized from ethanol to give colorless cubes, m. p. 174~176° (decomp.), yield 2.8 g. This was decided to be *cis*-isomer.

Anal. Calcd. for $C_{15}H_{11}O_2SCl$: C, 61.86; H, 3.79. Found: C, 62.02; H, 3.90.

The mother liquid was evaporated. The residue was dissolved in water. The aqueous solution was acidified to produce precipitation. The precipitate was recrystallized from alcohol to give 5.5 g of crude crystals. A solution of these crude crystals in 40 ml of alcohol containing 1.2 g of potassium hydroxide was kept in an ice-box overnight. The resulting deposit was discarded. The filtrate was evaporated and the residue was suspended in water and filtered. The filtrate was acidified in order to bring about precipitation. The precipitate was crystallized from acetone repeatedly to give plates; m. p. 172~173° (decomp.), yield 2.1 g.

Anal. Calcd. for $C_{15}H_{11}O_2SCl$: C, 61.86; H, 3.79. Found: C, 61.57; H, 3.90.

This was determined to be *trans*-isomer.

Methyl β-(*p*-acetamidophenyl) mercaptocinnamate (IV). To sodium alcoholate, which had been prepared from 1.6 g of metallic sodium and 30 ml of absolute ethanol, was added 11.5 g of purified *p*-acetamidothiophenol. To this mixture was gradually added 11 g of methyl phenylpropiolate (IX) under cooling in ice. Exothermal reaction occurred instantly and a white precipitate was produced. After standing at room temperature for fifteen minutes, the reaction was completed on a boiling water-bath for ten minutes. After cooling, the mixture was poured onto 300 ml of ice-water. The yellowish precipitate was collected by filtration, washed with water and recrystallized from ethanol to give slightly yellowish plates, m. p. 206~207°, yield 13.3 g. No different substances were found from the mother liquid after recrystallization.

Anal. Calcd. for $C_{18}H_{17}NO_3S$: C, 66.06; H, 5.20; N, 4.28. Found; C, 66.54; H, 5.68; N, 4.07.

β-(*p*-Acetamidophenyl) mercapto-*cis* and *trans*-cinnamic acids (V, VI). A mixture of 10.2 g of purified IV (m. p. 206~207°) and 2 g of potassium hydroxide in 150 ml of 95% ethanol was refluxed for three and a half hours. After cooling, the reaction mixture was poured into one liter of ice and water. The aqueous solution was filtered and acidified to give precipitation on cooling with ice. The precipitate was collected by filtration and dried in vacuo after being washed with cold water, m. p. 196° (decomp.), yield 9 g. It was recrystallized from 150 ml of ethanol to give colorless plates and

8) All melting points are uncorrected.

traces of needles. The crystals were collected by filtration. After the filtrate was concentrated to a half of its volume and refrigerated, a mixture of plates and needles were obtained again. Both were suspended in ethanol, and the light needles were mechanically separated by decantation from plates fixed at the bottom of a beaker. The mechanical separation and recrystallization were repeated to give plates (VI), m. p. 202° (decomp.) and needles (V), m. p. 218° (decomp.).

Anal. Calcd. for $C_{17}H_{15}NO_3S$; C, 65.17; H, 4.79; N, 4.47. Found from VI: C, 65.12; H, 4.76; N, 4.65.

The needles were further purified. A mixture of the needles and an equivalent of sodium hydroxide in alcohol were refluxed for eight and a half hours, diluted water and acidified to give a precipitate. The precipitate was recrystallized from alcohol to give needles, m. p. 226° (decomp.).

Anal. Calcd. for $C_{17}H_{15}NO_3S$: C, 65.17; H, 4.79; N, 4.47. Found; C, 65.45; H, 4.88; N, 4.63.

6-Chlorothioflavon (VII). This compound was prepared from II and III respectively in a way similar to the procedure of Ruhemann[1]. To a suspension of 1 g of II in benzene was added 0.8 g of phosphor pentachloride at room temperature. The mixture became clear orange and then color changed to green. After it was allowed to stand for forty-five minutes, the mixture was warmed gently on a water-bath for five minutes. The color changed again to yellowish orange. On cooling in ice, 0.6 g of aluminium chloride was added to the mixture. The mixture was allowed to stand for one hour and then poured into ice-water. Insoluble material was collected by filtration and recrystallized from acetone to give colorless needles, m. p. 189~190°, yield 0.6 g.

Anal. Calcd. for $C_{15}H_9SOCl$: C, 65.93; H, 3.29. Found: C, 66.15; H, 3.33.

This was produced in the same yield from III by the same procedure except that the color of the reaction mixture did not change in the process of chlorination.

6-Acetamidothioflavon (VIII). A mixture of 100 mg of V and 200 mg of phosphor pentachloride in 5 ml of dry benzene was kept at room temperature for half an hour, then at 55° for another half hour. To the mixture was added 100 mg of aluminium chloride under cooling with ice. After standing in an ice-bath for half an hour and at room temperature for fifty minutes, the mixture was poured into ice-water containing alkali. The insoluble material was collected by filtration, washed with water, and dried. This crude thioflavon weighed 50 mg. The alkaline filtrate was acidified to give a precipitate of acid fraction. The crude thioflavon was recrystallized from dioxan to give yellowish needles, m. p. 262°.

Anal. Calcd. for $C_{17}H_{13}NO_3S$: C, 69.15; H, 4.41; N, 4.75. Found: C, 68.74; H, 4.56; N, 4.86.

This compound was also produced in almost the same yield from VI in the same manner as V.

Ring closure under mild condition. a) β-(*p*-Chlorophenyl) mercaptocinnamic acids. To a mixture of 0.5 g of III in 5 ml of acetic anhydride was added 0.05 ml of concentrated sulfuric acid on cooling in ice. This was kept for twenty-four hours at room temperature, and warmed on a water-bath for fifteen minutes and then poured into ice-water. Forty mg of insoluble material was obtained and was recrystallized twice from acetone to give needles, melting at 177~180° alone and on admixture with the authentic sample of 6-chlorothioflavon (VII).

By similar treatment, 45 mg of crystals was obtained from II, but the m. p. was higher than 200° and depressed on admixture with an authentic sample of 6-chlorothioflavon (VII).

b) β-(*p*-Acetamidophenyl) mercaptocinnamic acids. Friedel-Craft's reaction was applied in the ice cooling procedure for one hour after chlorination with phosphor pentachloride had been completed. One-fifth g of VI gave 35 mg of 6-acetamidothioflavon (VIII), which was identified by a mixed melting point determination. However, V gave only a trace of the product and was almost entirely recovered.

Determination of pK values[9]. As the acids II and III are not soluble in water, 80% ethanol was used as the solvent. Alcoholic solutions from 10 to 20 mg of II or III were bubbled with a stream of nitrogen and titrated with dilute sodium hydroxide solution (0.075 N) in 80% alcohol. Apparent pH was measured by a glass electrode pH meter.

According to Henderson's equation, i. e., $pH = pKa + \log_{10}$ [salt]/[organic acid], pKa is tha pH at the state of half neutralization. Although this equation may not be applicable to the alcoholic solution, the apparent pH at this point may give a relative value of pKa. Therefore, only the difference of these values for each of the acids is given in the Table.

Spectrometrical determination of *cis-trans* mixture of β-(*p*-acetamidophenyl) mercaptocinnamic acid. The ratio of extinction of alcoholic solution at 259 mμ to that at 276 mμ was plotted against composition of the mixture (Fig. 6). A linear relationship was found. The ratio

9) R.P. Linstead, J.A. Elvidge and M. Whalley, A Course in Modern Techniques of Organic Chemistry, p. 159 (1955), Butterworths Scientific Publications, London.

was 0.79 at 100% VI and 1.33 at 100% V.

Saponification of methyl β-(*p*-acetamidophenyl) mercaptocinnamate (I). A mixture of 120 mg of the purified ester and 25 mg of potassium hydroxide in 7 ml of 95% ethanol was refluxed for three and a half hours, and was poured into ice-water. The aqueous solution was acidified to give a precipitate, which was collected by filtration, washed with water, and dried to give 100 mg of acid. It was then dissolved in ethanol and subjected to spectrometry. The E_{259}/E_{276} ratio was 0.84. This showed it to be a mixture of 90% *trans*-acid and 10% *cis*-acid.

Isomerization of β-(*p*-acetamidophenyl) mercaptocinnamic acids. a) A mixture of 30 mg of VI and 0.1 ml of N-sodium hydroxide in 2 ml of ethanol was refluxed for four hours and poured into ice-water. The precipitate produced by acidication was collected by filtration and dissolved in ethanol after washing with water. This was subjected to spectrometrical determination. The E_{259}/E_{276} ratio was 0.84. This showed that 10 percent of it is isomerized to V.

b) In the procedure to form 6-acetamidothioflavon (VIII) by the action of aluminium chloride upon the chloride of V or VI, the acid fraction (see above) separated from thioflavon was subjected to spectrometrical determination. The E_{259}/E_{276} ratio of the acid fraction from *cis*-acid (V) was 0.92. This showed it to contain 76% *trans*-acid (VI). The absorption ratio of that from *trans*-acid (VI) was 1.03, which corresponded to a content of 44% *cis*-acid (V).

Acknowledgement. The authors wish to express their thanks to Prof. T. Taguchi of Pharmaceutical Institute, Kyushu University for his useful advice and discussions in concern of stereochemical aspects. Indebts are also due to the Service Center of Microanalyses of Kyushu University for the microanalyses and also to Mr. Yano of Pharmaceutical Institute, Kyushu University for infrared spectra.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 481~489, 1960]

Studies on the Utilization of Levulinic Acid

Part II. Promotive Action of Acid Oximes on the Excretion of Radioactive Cesium*

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Received December 9, 1959

The single injection of levulinic acid oxime (250 mg/rat) or α-ketoglutaric acid oxime (250 mg/rat) on rats, carrying radioactive cesium, promoted both urinary and fecal excretion of this radionuclide. The administration of levulinic acid oxime (sodium salt) decreased the cesium retention by liver. The administration of the oxime did not have influence on the urinary excretion of sodium and potassium in normal rats. The toxicity of the oxime was low. The LD_{50} of α-ketoglutaric acid oxime was 3500 mg/kg (mice, intraperitoneally). (The LD_{50} of levulinic acid oxime has already been indicated as 2040 mg/kg (mice, intravenously)[1].

INTRODUCTION

In 1945, Hamilton[2] observed approximately 100% absorption of radioactive cesium after ingestion, a low skeletal uptake, and a high accumulation in the soft tissues. Concerning the excretion of the administered radioactive elements, several experiments[2~7] have been carried out. The excretion of the cesium is slow, requiring approximately 7~10 days to excrete 50~70 per cent of the administered dose, and 64 days to excrete 99 per cent[4].

Radioactive cesium and strontium are known to be dangerous elements in the fission product. A relatively high accumulation of the strontium is observed in the skeleton, and the cesium is highly accumulated in the soft tissues[3,8], especially in the muscle. The physiological effects of these elements remaining in the tissues shall not be overseen[8], as each of them have a relatively long half-life, either physically or physiologically. Although studies to promote the excretion of radioactive strontium by administrating drugs, such as chelating agents, have been carried out by many authors, so far, there are few similar studies on th excretion of the radioactive cesium.

(a) Cesium, an alkali metal, has been reported to behave similarly to sodium, potassium, and rubidium. Mac. Leod and Snell[9], Love and Burch[10] observed the biological similarity of cesium to potassium in both bacteria and human erythrocytes, respectively. The effect of dietary potassium on cesium excretion was examined by Mraz and Patrick[11], Williams and Patrick[12],

* Some of the data have been presented at the meeting of the Agricultural Chemical Society of Japan, held at Tokyo, Sept. 27, 1958.

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1) K. Takamiya, GANN, **50**, 267 (1959); *J. Antib.*, Ser. A, **9**, 222 (1956).

2) J.G. Hamilton, MDDC 1062, Feb. 2, 1945; MDDC 1160, June 11, 1947; *Radiology*, **49**, 325~343 (1947).

3) S.L. Hood and C.L. Comar, ORO-91 (1953); *Arch. Biochem. and Biophys.*, **45**, 423~433 (1953).

4) K.G. Scott et al., MDDC 1275 (1947).

5) F.R. Mraz and H. Patrick, *Federation Proc.*, **15**, 565 (1956).

6) T. Watari, Nippon Acta Radiol., 1502~1507 (1958).

7) H. Yoshikawa et al., Ann. Rep. Co-ope. Res. (Radiation) M. Educ. (1957).

8) T. Watari, Nippon Acta Radiol., 1480~1501 (1958).

9) R.A. Mac. Leod and E.E. Snell, *J. Bacteriol.*, **59**, 783~792 (1950).

10) W.D. Love and G.E. Burch, *J. Lab. Clin. Med.*, **41**, 351~362 (1953).

11) F.R. Mraz and H. Patrick, *Proc. Soc. Exptl. Biol. Med.*, **94**, 409~412 (1957).

and others. The increased excretion of the radioactive cesium was observed by administration of potassium[11,12] (and sodium[15]). The supplementation of a simplified died with some feed stuffs[5,13] (oat hulls, or others) increased the total excretion of cesium-134.

(b) Mraz and Patrick[11,16] fed rats with the following materials to increase their fecal excretion of cesium-134. The materials employed were vermiculite, bentonite, (alfalfa, beet pulp[12], bone charcoal, and Amberlite IRC-50). The former two increased fecal excretion of cesium-134, but the volume of the gut of rats was a limited factor.

(c) Suzuki[17] examined the effect of diuretics on cesium excretion, and found that they did not influence the retention of the radioactive element. Cesium is excreted by the kidneys, and parathyroid extract causes diuresis[18]. The following hormones have been examined as whether they could influence the retention or not. These hormones, such as cortisone[12,18], did not show a clear increase in cesium excretion. Machida et al. assumed aldosterone and DOCA[14] to be most suitable for this purpose[19]. Other hormones examined[20] were throxine, adrenaline, noradrenaline, insuline, tetrosterone, estradiol, and desoxycorticosterone[21], etc.. Vitamine D_2 and glucose were also examined and were found to rather decrease the total excretion of cesium.

(d) A sodium and calcium salt of citric acid has been indicated by Ogawa to promote the excretion of radioactive elements, cesium-137, strontium-90, and zirconium-95.

12) L.G. Williams and H. Patrick, *Arch. Biochem. and Biophys.*, **70**, 464~468 (1957).
13) F.R. Mraz and H. Patrick, *J. Nutrition*, **61**, 535~546 (1957).
14) S.A. Threefoot, G.E. Burch and C.T. Ray, *J. Lab. Clin. Med.*, **45**, 313~322 (1955).
15) F.R. Mraz, M. Le Noir, J.J. Pinajian and H. Patrick, *Arch. Biochem. Biophys.*, **66**, 177~182 (1957); E. Ogawa et al., *Kitakanto Med. J.*, **8**, 258~263 (1958).
16) F.R. Mraz and H. Patrick, *Arch. Biochem. and Biophys.*, **71**, 121~125 (1957).
17) S. Suzuki, *Kitakanto Med. J.*, **8**, 264~271 (1958).
18) F. Mraz, M. Le Noir, J. Pinazian and H. Patrick, *Arch. Biochem. and Biophys.*, **63**, 73~76 (1956).
19) J. Machida,, *Kitakanto Med. J.*, **8**, 272~281 (1958).
20) J. Machida, *ibid.*, **8**, 282~287 (1958).
21) R.I. Dorfman and A.M. Pott, *Proc. Soc. Exptl. Biol. Med.*, **72**, 702 (1949).

In the course of investigation of the biological effects of levulinic acid oxime, as a practical use of levulinic acid, which is an agricultural waste, the promotive action of the levulinic acid oxime, and α-ketoglutaric acid oxime on cesium excretion was observed, and is described in this paper.

EXPERIMENTAL

Levulinic acid oxime[22], m.p. 97°C, α-ketoglutaric acid oxime[23], m.p. 140~141°C (dec.), or ethylenediamine tetraacetic acid (EDTA-2Na) was dissolved into physiological saline and neutralized to pH 7 by sodium hydroxide or potassium hydroxide, respectively.

Determination of the excreted cesium in urine of rats.

Each rat, weighing 170~270 g body weight, was administered 1 ml of the solution (pH 7) intraperitoneally, containing a radioactive cesium of 8.8 μc of cesium-134, or 10 μc of cesium-137. Urines were collected after every 24 hours and washed into a scaled flask, then filled up to 25 ml with water. A 0.5 ml aliquot of this test solution was evaporated to dryness on a stainless-steel dish, and determined its count per minute (cpm) with a Geiger-Müller counter (Scientific Research Institute, Counter Model-100-ASA4). The distance from the mica window (mica: 2.3 mg/cm$_2$) to the sample dish was 1.3 cm in length.

The mice were supplied a commercial type ration, Oriental compressed diet.

Determination of fecal excretion of the cesium.

The feces were collected every 24 hours. After digestion with concentrated nitric acid, the fecal solution was diluted to 50 ml. A 0.5 ml aliquot of the diluted solution was counted its cpm after drying on the same sample dish. The distance from the mica window to the sample dish was 1.3 cm in length.

Determination of the accumulated cesium-131 in organs of a mouse.

The sample solution, 0.1 ml (pH 7) containing 8.8 μc of cesium-134, was injected intraperitoneally into mice weighing 15~18 g body weight. Sodium salt of levulinic acid oxime (5 mg, or 10 mg of the oxime per mouse) was injected daily into the peritoneal cavity 4 times, initiating 6 or 24 hours after administration of radioactive cesium. At 24 hours after the last injection, the mice were sacrificed and dissected to collect their kidneys,

22) P. Rischbieth, *Ber.*, **20**, (2670).
23) M.M.E.E. Blaise and J. Gault, *Bull. Soc. chim. France*, IV, **9**, 457.

testis, livers, spleens, and muscle. Each organ was dried at 100°C, and ashed at about 500~600°C to analyze for cesium-134. The distance from the mica window to the sample dish was 3.8 cm in length. Each group consisted with ten mice.

Determination of the excreted sodium and potassium in urine of rats.

Each rat of 270 g body weight was put to use. Urines were collected at every 24 hours, and the amount of excreted sodium and potassium were measured, respectively, with a "Lange Flammenphotometer Model 4". The commercial type ration, supplied, contained 0.474% of potassium and 0.300% of sodium.

RESULTS

Acute toxicity of α-ketoglutaric acid oxime.

The solution containing each dose of α-ketoglutaric acid oxime was administered intraperitoneally into mice, weighing 18~20 g body weight. The LD_{50} was 3.5 g/kg (mice, intraperitoneally), as shown in Table I.

TABLE I. TOXICITY OF α-KETOGLUTARIC ACID OXIME.

LD_{50}=3.5 g/kg (mice, intraperitoneally)

dose (g/kg)	Number of mice dying			
	1st day	2nd day	3rd day	9th day
5	4/5	5/5	5/5	5/5
3.5	1/5	2/5	2/5	2/5
2	0/5	0/5	0/5	0/5

Influence of oximes on the metabolism of radioactive cesiums.

The solution containing 8.8 μc of cesium-134 (Cs-134-p) was neutralized with sodium hydroxide to pH 7, and administered intraperitoneally to each rat. The urinary excretion was maximum at the first 24 hours after administration, decreasing almost exponentially. After an elapse of several days, when daily excretion of cesium became approximately constant, the solution containing levulinic acid oxime (sodium salt) was injected into the peritoneal cavity. The increase of urinary excretion of radioactive cesium was observed within the following 48 hours (Fig. 1). The same effect was also observed with the injection of α-ketoglutaric acid oxime (sodium salt), as shown in Fig. 2. As controls, physiological saline or EDTA (sodium salt) was injected. These reagent did not afford detectable increase of urinary excretion of cesium-134 (Fig. 2).

In the case of cesium-137 (Fig. 3), the same promotive effects of cesium excretion were caused by injection of levulinic acid oxime and α-ketoglutaric acid oxime. Both fecal and urinary excretion of cesium-137 were increased by the administration of these two oximes. DOCA did not afford detectable influence on the excretion in case of a dose of 5 mg/rat.

The influence of levulinc acid oxime (sodium salt) on cesium-134 retention by the organs was examined with mice. The administration of levulinic acid oxime decreased the cesium retention caused by the liver, but not in case of muscle, testis, spleen, and the kidneys. The results are shown in Fig. 4.

Influence of oximes on the urinary excretion of sodium and potassium by rats.

The urinary excretion of sodium and potassium by normal rats, carrying not the radioactive cesium, was about 80~120 and 160 mg/day, respectively. The injection of levulinic acid oxime (sodium salt) caused an increase in the urinary excretion of sodium, while no increase was observed in case of potassium. On the contrary, the injection of the oxime (potassium salt) increased potassium excretion, but did not have effect on sodium excretion. Also, injection of α-ketoglutaric acid oxime (sodium salt) did not cause any increase in excretion of urinary potassium (Fig. 5), whereas it increased the sodium excretion.

DISCUSSION

The urinary excretion of radioactive cesium is indicated by several authors[3~6,18] to be many times as much as that of the fecal excretion. Furthermore, the daily excretion of urinary cesium was indicated to be independent of the total amount of daily urine. Thus, the effect of oximes on cesium excretion was first examined with rat's urine. The urinary excretion decreased daily almost exponentially. A single injection of sodium salt of levulinic acid oxime or α-

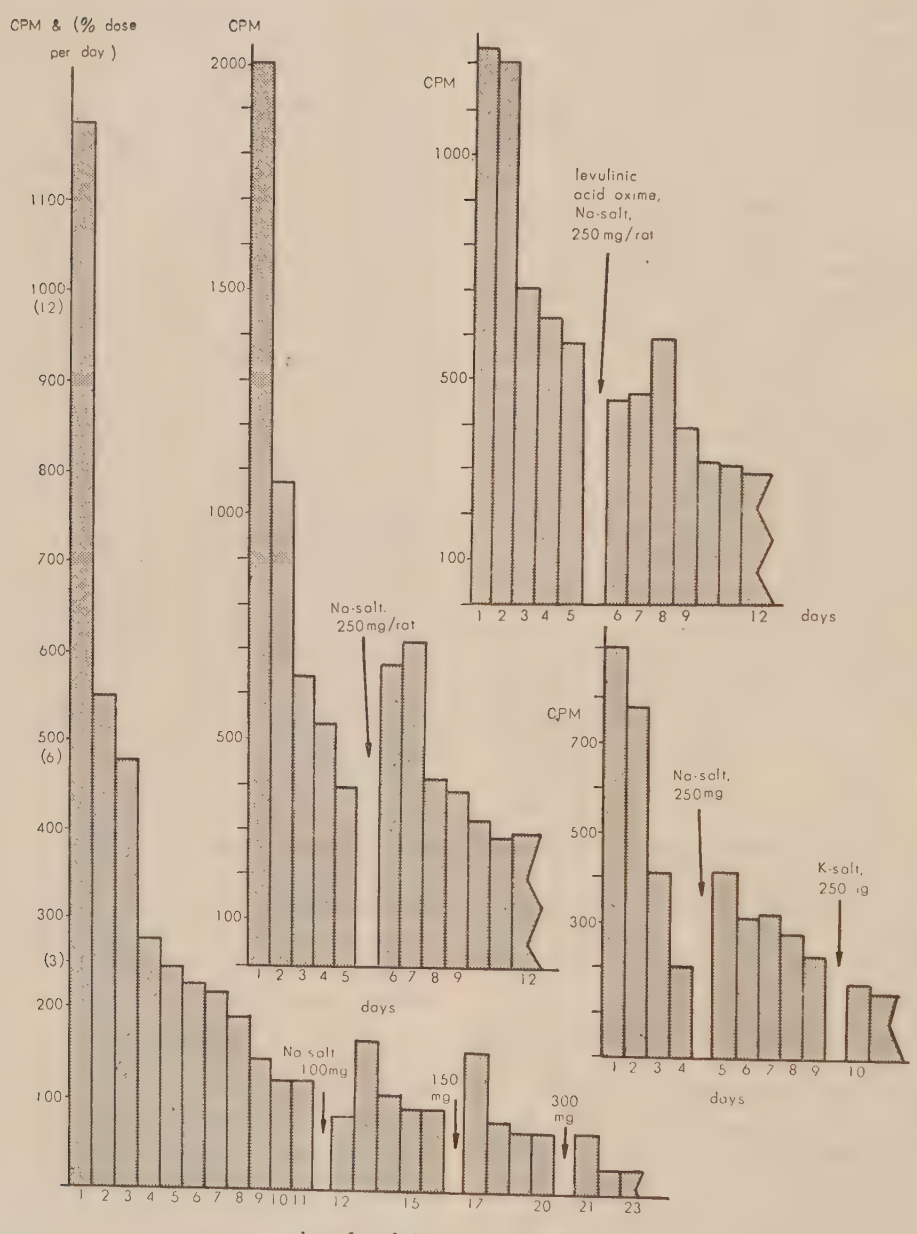


FIG. 1. Influence of Levulinic Acid Oxime on the Urinary Excretion of Cesium-134 by Rats.

The excretory examinations were investigated several times with using about thirty rats. Figures 1, 2 and 3 show some typical patterns.

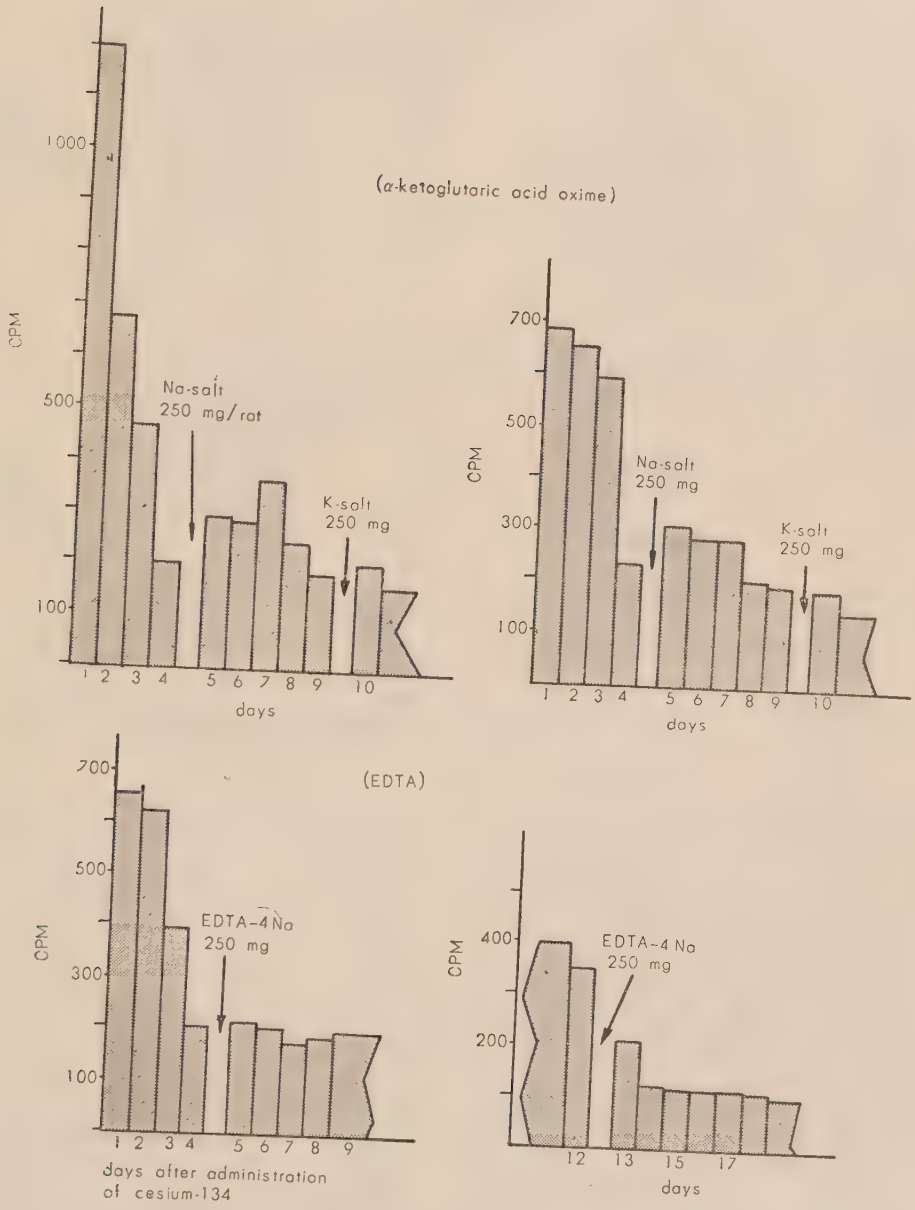
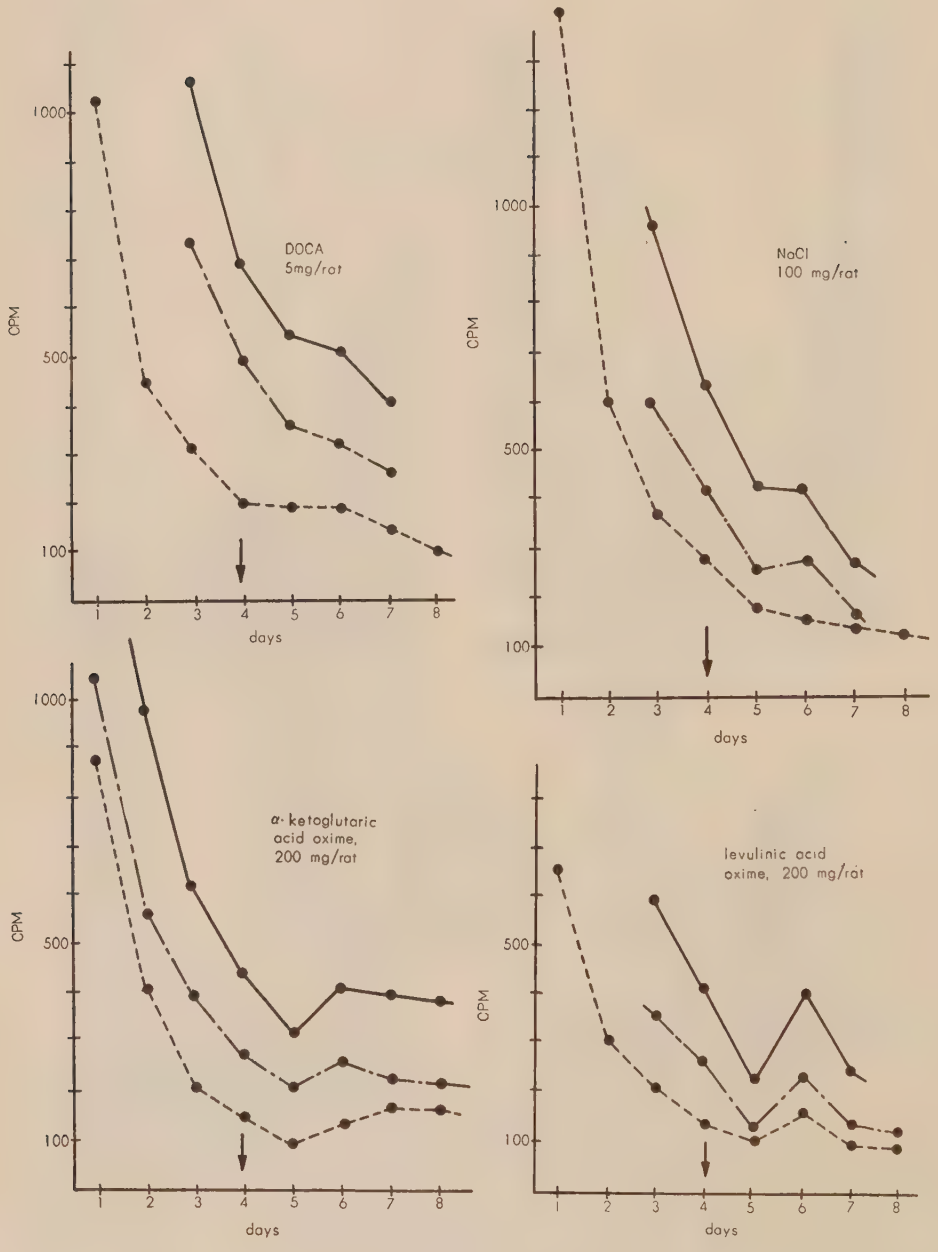


FIG. 2. Influences of α-Ketoglutaric Acid Oxime or Ethylenediamine Tetraacetic Acid on the Urinary Excretion of Cesium-134 by Rats.



days after administration of cesium-137

FIG. 3. Influences on the Urinary Excretion of Cesium-137 by Rat.
—— Total excretion —·—·— Fecal excretion - - - Urinary excretion

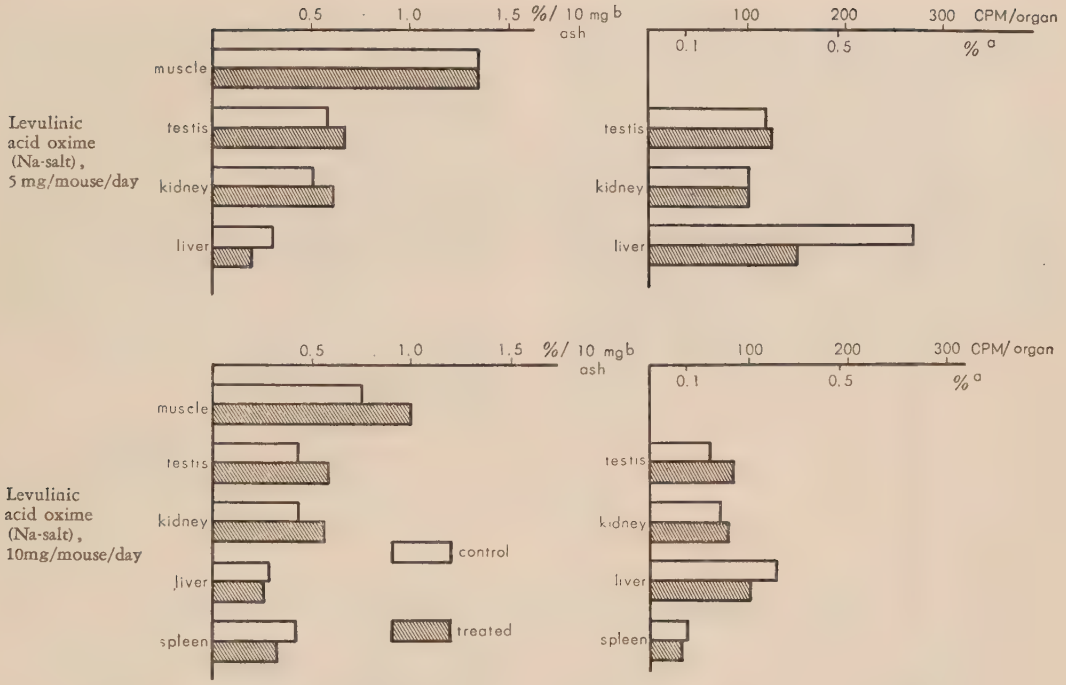


FIG. 4. Influence of Levulinic Acid Oxime (Na-salt) on the Metabolism of Cesium-134.

a: Expressed as per cent of the administered dose of Cs-134 per total ash of each organ.
b: Expressed as per cent of administered dose per 10 mg ash of each organ.

ketoglutaric acid oxime was performed several days after cesium-134 administration. An increased excretion of uinary cesium-134 was observed, as shown in Figs. 1 and 2.

The same increased excretion of the urinary and fecal excretion of cesium-137 were also observed as shown in Fig. 3.

The findings that sodium administration might increase cesium excretion have been demonstrated by Mraz, Ogawa, and others[15]. The promotive action of levulinic acid oxime (sodium salt) on cesium excretion might be responsible to the promotive action of sodium itself. Sodium chloride (100 mg/rat, containing about 60 mg/rat of sodium, Fig. 3), or EDTA solution (containing 250 mg/rat of EDTA-2 Na, 63 mg/rat of sodium, Fig. 2) was injected intraperitoneally into rats carrying radioactive cesium. However, they did not show distinct effects on cesium excretion. The promotive action of the oxime solution (containing 250 mg/rat of the oxime, about 1~1.6 mg/rat of sodium) may be due to that of the oxime itself.

The parathyroid extract has been reported to slightly increase cesium excretion with its consecutive injections, initiating just after cesium administration. Though it is still somewhat obscure, cortisone and other hormones are reported to increase the urinary excretion of potassium[24]. Potassium and cesium are reported to behave similarly. Levulinic acid oxime was found to influence the cesium retention. Then, effects of the oximes on urinary excretion of sodium and potassium were investigated with normal rats, carrying not the radioactive cesium, as shown in Fig. 5. The intraperitoneal injec-

24) G.J. Hamwi and R.F. Goldberg., *J. Am. Med. Assoc.*, **159**, 1958 (1955);
F.A. Hartman and C.G. Toby., *Endocrinology*, **22**, 207 (1938);
D.J. Ingle et al., *ibid.*, **39**, 32 (1946); **46**, 510 (1950);
J.P. Peters, *J. Lancet*, **73**, 180 (1953).

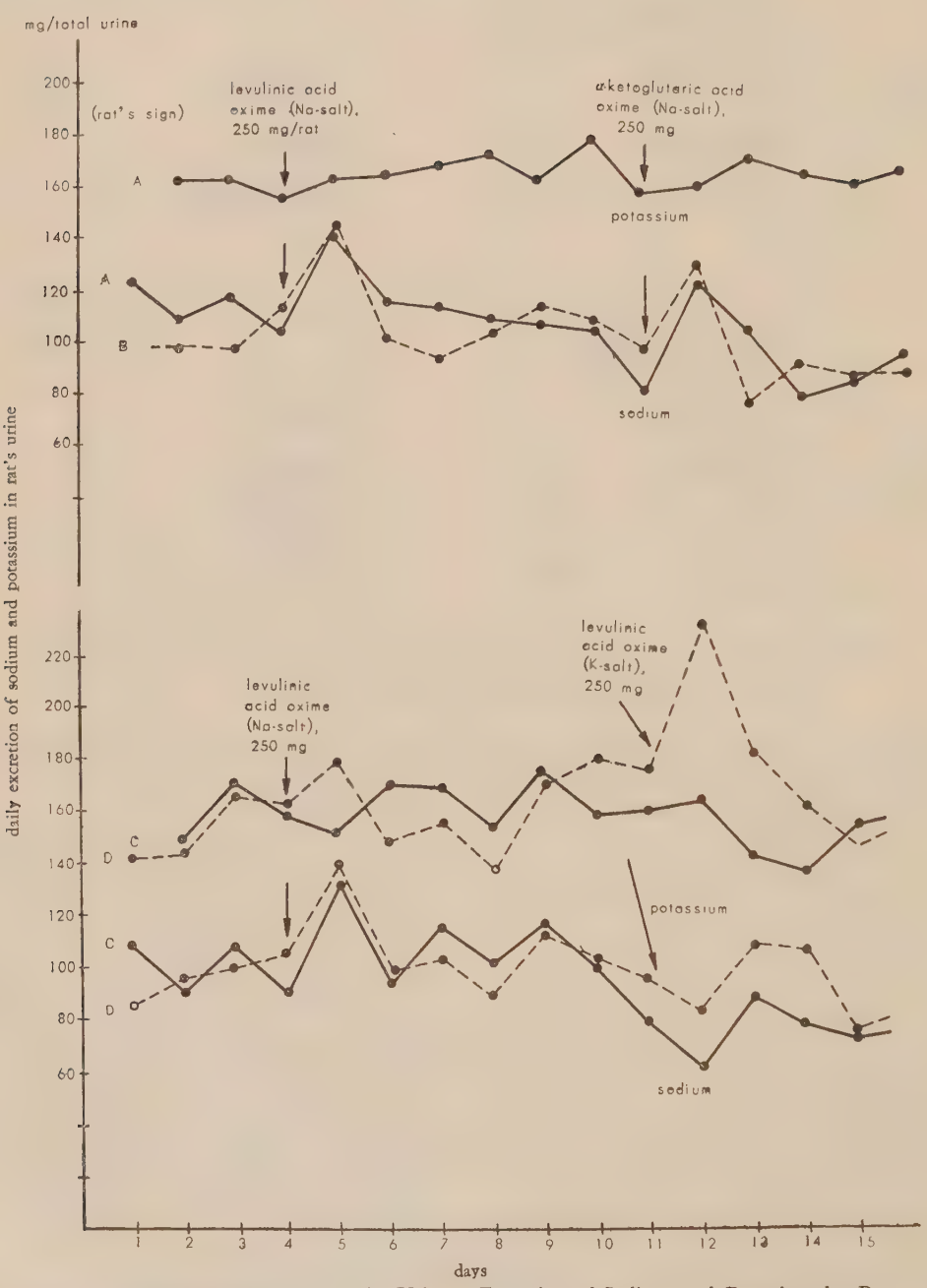


FIG. 5. Influence of Oximes on the Urinary Excretion of Sodium and Potassium by Rats.

tion of levulinic acid oxime did not influence the urinary excretion of sodium and potassium in normal rats.

The sodium salt of levulinic acid oxime was injected into mice in order to measure the remaining cesium-134 in mice organs. The decrease of cesium-134 retention was observed in liver, but not in muscle, testis, spleen, and the kidneys. This point would be noteworthy in our future study.

The two oximes examined in this paper were almost non-toxic. LD_{50} of the oximes were 2040 mg/kg (levulinic acid oxime, mice, intravenously), and 3500 mg/kg (α-ketoglutaric acid oxime, intraperitoneally), respectively.

Acknowledgements The author wishes to express his sincerest thanks to Prof. Y. Sumiki for his encouragement and guidance through these experiments. He is thankful to Mr. Ko-ling Yeh and Miss H. Toyozumi, Departmen of Agricultural Chemistry, University of Tokyo, for their contributions in the course of this experiment.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 489~496, 1960]

Betaine as a Growth Factor for *Pediococcus soyae*

Part VIII. Studies on the Activities of Bacteria in Soy Sauce Brewing

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Received December 9, 1959

Besides P-factor[1], *Pediococcus soyae*[2,3] requires betaine (glycine-betaine) as a specific growth promotant. The maximal growth is obtained with the supplementation of both betaine and P-factor to the synthetic medium, while betaine only gives the half-maximal growth. When the organism is cultured in 18% salted media, the addition of both betaine and P-factor is essential for the occurrence of growth. Thus, betaine is requisite for *Ped. soyae* as a growth promoting factor and also as a factor bestowing the osmotelerant ability.

Pediococcus soyae is characterized by three eminent specificities, namely, by its acid-sensitivity[2], by its osmotolerant character to grow even in 24~26% salted media[1,2], and by its unique nutritional requirements[1]. This organism does not grow in the ordinary syntheic medium for lactic acid bacteria, and requires two additional growth factors. One of them, which was provisionally named as P-factor in the preceding papers, is found to be included in partial hydrolysates of Hammersten's milk casein prepared with hydrochloric acid or various crystalline proteinases. The other, S-factor, is found to be present in Difco's yeast extract, beef extract, heart infusion broth, in soy sauce, and in the water extract of *Aspergillus soyae* mycelium. The maximal growth is, however,

1) Kenji Sakaguchi, This Bulletin, **23**, 438, 443 (1959).
2) Kenji Sakaguchi, This Bulletin, **22**, 353 (1958); **23**, 22, 100 (1959); *Rep. Noda Inst. Sci. Res.*, **2**, 40 (1958).
3) K. Yamazato and H. Iizuka, *J. Agr. Chem. Soc. Japan*, **33**, 379, 383 (1958); *J. Gen. Appl. Microbiol.*, **5**, 58 (1959).

obtained by the addition of both of the two factors; P-factor alone, giving rise to only the half-maximal growth.

This report deals with the betaine requirement of *Ped. soyae* and the fact that the S-factor and betaine are probably identical. Some results obtained from the examination of specific nutritional requirements of the organism under salt-tolerant conditions are also described.

TABLE I. COMPOSITION OF THE BASAL-SYNTHETIC MEDIUM

DL-Ala	200 mg/l
DL-Asp	400
L-Glu	500
L-Arg·HCl	200
L-Lys·HCl	200
L-His·HCl	100
DL-Ileu	200
DL-Met	200
DL-Phe	200
L-Pro	100
DL-Thr	200
L-Tyr	100
DL-Val	200
DL-Try	100
L-CyS	100
DL-Ser	100
Gly	100
L-Leu	100
Glucose	20 g/l
Na-actate·$3H_2O$	33
K_2HPO_4	500 mg/l
KH_2PO_4	500
$MgSO_4 \cdot 7H_2O$	200
$FeSO_4 \cdot 7H_2O$	10
$MnSO_4 \cdot 4H_2O$	10
NH_4Cl	3 g/l
Adenine sulphate	10 mg/l
Guanine HCl	10
Uracil	10
Xanthine	10
Riboflavine	2
Thiamine·HCl	1
P.A.B.A.	1
Pyridoxine·HCl	1
Ca-*dl*-Pantothenate	1
Nicotinic acid	1
Biotin	10 γ/l
Folic Acid	10
Choline	2 mg/l
Inositiol	2
Leucovorin	10 γ/l

pH 7.0, Pyridoxal is omitted.

METHODS

I. Assay procedure.

The basal synthetic medium employed was essentially the same as that described by Snell and others[4] with the following alteration. The supplementation with choline 2 mg/l, inositol 2 mg/l and leucovorin 10 γ/l was made. Pyridoxal was omitted because it is replaceable with pyridoxine. L-Leucine and L-methionine were each substituted by two-fold amounts of DL-leucine and DL-methionine.

Stock cultures of *Ped. soyae* strain d 2 were maintained on 7% salted yeast extract-peptone-glucose-agar stabs and transferred monthly. Inocula were grown in the following medium; yeast extract (Difco) 0.3%, Polypeptone (Takeda) 1.0%, glucose 1.0%, sodium chloride 0.5%, sodium acetate trihydrate 3.3% and dipotassium phosphate 0.5%, pH 7.0. After four days of incubation at 30°C, the cells were centrifuged, washed four times with 0.9% potassium chloride solution, and diluted to give a final optical density of 0.07 at 660 mμ. Five-ml culture fluids containing appropriate supplements were prepared in the usual manner[1], autoclaved for fifteen minutes at 120°C, cooled, incubated with one drop per tube of the cell suspension described above and incubated at 30°C. Growth was estimated photometrically at 660 mμ in a spectrophotometer. When the cells were cultured in 18% salted media, inocula were grown in 18% salted broth, and washed with a solution containing 18% sodium chloride, 1.0% glucose, and 3.3% sodium acetate trihydrate, pH 6.0. This solution is favourable for keeping the viability of the inocula grown in salted media.

RESULTS

I. Requirements of both P- and S-factors in salted environments.

When cultured in unsalted media, *Pediococcus soyae* shows half-maximal growth with the supplementation of P-factor alone, and maximal growth is observed by the addition of both P- and S-factors[1]. In 18% salted media, however, nothing was known on nutritional requirements. The results shown in Fig. 1 indicate that, under

4) E.E. Snell, *Nutrition Abstr. & Rev.*, **16**, 497 (1946~47); G. Tamura et al., *J. Agr. Chem. Soc. Japan*, **26**, 464, 474 (1952).

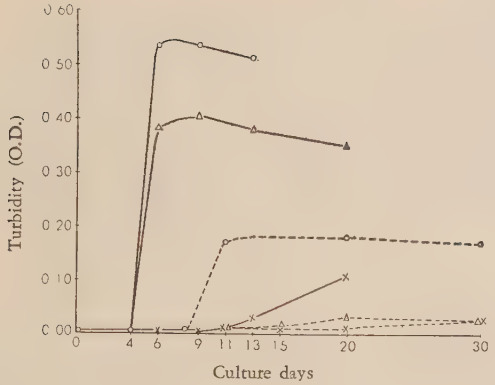

FIG. 1. Nutritional Requirements of *Ped. soyae* in Salted and Unsalted Media.

—×— Synthetic medium
—△— +Polypepton 1.0%
—○— +Polypepton 1.0% & Yeast extract 0.3%
} NaCl 0.02%

---×--- Synthetic medium
---△--- +Polypepton 1.0%
---○--- +Polypepton 1.0% & Yeast extract 0.3%
} NaCl 18.0%

the salted environment, no growth was observed by the mere addition of P-factor (Polypepton Takeda 1.0%) and the addition of both P- and S-factors was absolutely needed for the occurrence of growth. In the cultures with heavily salted media a longer lag period of more than six days resulted, and the final growth was also poorer.

II. Detection of the effective substance amongst various vitamins and growth stimulants.

Seventy-six vitamins and growth stimulants[5~13] were collected and their effects on *Ped. soyae* were examined. The inspection was made after four days' culture in unsalted media, and after fifteen days' culture in 18% salted media supplemented with P-factor. Amongst various growth promotants only betaine was found to be effective in both salted and unsalted media.

III. Optimum concentration of betaine hydrochloride.

To the unsalted synthetic medium, betaine

TABLE II. EFFECT OF GROWTH PROMOTANTS ON *Ped. soyae*

	μg/ml	NaCl 0.02%	NaCl 18% polypepton 1.0%
none		0.01	0.00
polypepton	10.000	0.53	0.05
polypepton+yeast-extract		0.76	0.14
polypepton+beef-extract		——	0.15
betaine HCl	30	0.45	0.00
betaine HCl+polypepton		0.67	0.17
ergosterol	10	0.00	0.04
cholesterol	〃	0.00	0.02
progesterone	〃	0.00	0.02
α-tocopherol	〃	0.00	0.08
ATP	〃	0.03	0.04
FMN	〃	0.02	0.04
FAD	〃	0.03	0.05
DPN[14]	〃	0.02	0.05
TPN	〃	0.03	0.05
coenzyme A	〃	——	0.05
yeast RNA	〃	0.03	0.05
spleen DNA	〃	0.00	0.04
adenosine[15]	〃	0.02	0.05
adenylic acid	〃	0.01	0.04
deoxyadenosine	〃	0.01	0.05
deoxyadenylic acid	〃	0.01	0.04
cytidine	〃	0.01	0.04
cytidylic acid[16]	〃	0.03	0.04
deoxycytidine	〃	0.02	0.04
deoxycytidylic acid	〃	0.01	0.04
guanosine	〃	0.02	0.04
guanylic acid	〃	0.03	0.04
deoxyguanosine	〃	0.02	0.06
deoxyguanylic acid	〃	0.03	0.04
uridine	〃	0.04	0.05
uridylic acid[16]	〃	0.01	0.04
thymine	〃	0.01	0.06
thymidine[17,18]	〃	0.03	0.05
thymidylic acid	〃	0.04	0.04
Panvitan solution	1000	0.00	0.04
VB_{12}[19]	10	0.00	0.06

5) E.E. Snell, *Ann. Rev. Microbiol.*, **3**, 97 (1949).
6) W.A. Krehl and S.J. Kiao, *ibid.*, **5**, 129 (1951).
7) J.L. Stokes, *ibid.*, **6**, 29 (1952).
8) V.H. Cheldelin and T.E. King, *ibid.*, **7**, 113 (1953).
9) D. Hendlin, *ibid.*, **8**, 47 (1954).
10) E.L.R. Stockstad et al., *ibid.*, **9**, 111 (1955).
11) L.D. Wright, *ibid.*, **10**, 141 (1956).
12) B. Magasanik, *ibid.*, **11**, 221 (1957).
13) B.M. Guirard, *ibid.*, **12**, 247 (1958).
14) K. Kitahara and A. Ôbayashi, *J. Agr. Chem. Soc. Japan*, **33**, 497 (1959).
15) T. Fukami, Lecture given on July 4th, 1959 at the First Meeting of the Symposium on Microbioassay.
16) M. Ikawa and J.S. O'Bar, *J. Bact.*, **71**, 401 (1956).
17) E.A. Felton and C.F. Niven, *J. Bact.*, **65**, 482 (1953).
18) E. Kitay and E.E. Snell, *ibid.*, **59**, 727 (1950); **60**, 49 (1950).
19) I. Kusaka and K. Kitahara, *J. Agr. Chem Soc. Japan*, **33**, 703 (1959).

VK_3	10	0.00	0.07
nyacin amide	//	0.02	0.05
ascorbic acid	100	0.01	0.07
mevalonic acid (synth.)[20,21]	10	0.02	0.04
// (natural)	//	——	0.06
lipoic acid[22]	//	0.01	0.05
orotic acid[23,24]	//	0.03	0.05
oleic acid[25,26]	//	0.03	0.06
DL-desthiobiotine	//	0.01	0.03
DL-pantoyl lactone	//	0.03	0.05
taurine	//	0.03	0.05
tween 80[25,27]	200	0.04	0.05
N-acetyl glucosamine[28~30]	100	0.01	——
β-alanine	10	0.02	0.05
D-glutamic acid[31]	100	0.03	0.04
diiodotyrosine	10	0.01	——
thyroxine	//	0.02	——
insulin	100	0.01	0.07
glutamine	//	0.01	0.04
asparagine	//	0.01	0.04
glutathione	//	0.01	0.04
Pro-Leu anhydride[32]	//	——	0.03
Pro-Val anhydride[32]	//	——	0.03
Pro-Leu	//	——	0.03
Pro-Val	//	——	0.03
γ-amino butyric acid	//	0.01	0.04
oxaloacetic acid	100	0.01	——
Ba-glucose-6-phosphate	//	——	0.04
Ba-hexose-1,6-diphosphate	//	0.02	0.05
Na-β-glycerophosphate	//	0.01	0.05
dihydroxyacetone	//	0.01	——
fumaric acid	//	0.00	——
lactic acid	//	0.00	——
succinic acid	//	0.00	——
glycolic acid	//	0.00	——
malic acid	//	0.00	——
citric acid	//	0.01	——
DL-glyceraldehyde	//	0.01	——
Ba-phosphoglycerate	//	0.01	——
pimelic acid	10	0.01	0.04
L,L-diamino pimelic acid[33,34]	//	0.01	0.05
L,D-diamino pimelic acid	//	0.01	0.05
D,D-diamino pimelic acid	//	0.00	0.06
rutin	//	0.03	0.05

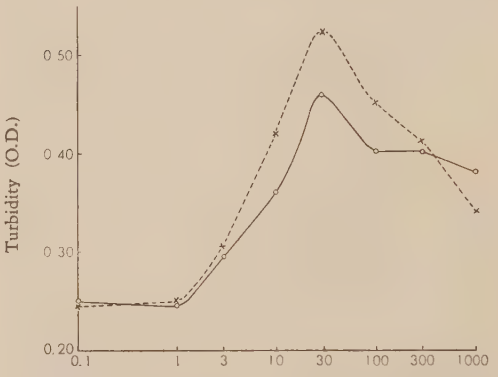

FIG. 2. Optimal Concentration of Betaine Hydrochloride.
——○—— 9 days incubated.
---×--- 10 days incubated.

hydrochloride was added in concentrations of 0.1, 1.0, 3.0, 10, 30, 100, 300, and 1000 mg/l and the growth response was measured after nine and ten days of culture. Fig. 2 shows that the best growth was obtained with the addition of 30 mg betaine hydorochloride per litre. However, even in this optimum concentration, the supplement of betaine alone resulted in the half-maximal growth. After nine days of culture, the synthetic media attained the final growth of 0.25 optical density, the supplement of 1.0% Polypepton 0.43, betaine hydrochloride 30 mg/l 0.46, while media added with Polypepton 1.0% and yeast extract 0.3% attained the optical density of 0.64. It is thus approvable that besides betaine, another growth factor is necessary for the maximal growth of *Ped. soyae*.

IV Effects of various amines and related substances with betaine.

20) G. Tamura, This Bulletin, **21**, 202, 394 (1957); **22**, 202 (1958); *J. Agr. Chem. Soc. Japan*, **32**, 701, 707, 778, 783 (1958).
21) D.E. Wolf et al., *J. Amer. Chem. Soc.*, **78**, 4499, 5273 (1956); **79**, 1486 (1957).
22) L.J. Reed et al., *Science*, **114**, 93 (1951).
23) I. Crawford et al., *J. Biol. Chem.*, **226**, 1093 (1957).
24) L.D. Wright et al., *Proc. Soc. Exptl. Biol. Med.*, **84**, 716 (1953).
25) Y. Tani and S. Tatsumi, *J. Ferment. Technol.*, **36**, 311, 317 (1958).
26) K. Hofmann, et al., *J. Biol. Chem.*, **228**, 349 (1957).
27) L. Gerschenfeld, *J. Am. Pharm. Ass. Ed.*, **42**, 187 (1953).
28) P. Georgy, R. Kuhn et al., *Am. J. Disease Children*, **84**, 484 (1952); **85**, 632 (1953).
29) R. Kuhn, *Angew. Chem.*, **64**, 493 (1952).
30) S. Pope et al., *Arch. Biochem. Biophys.*, **68**, 362 (1957).
31) S.A. Koser and J.L. Thomas, *J. Bact.*, **73**, 477 (1957).
32) Y. Koaze, This Bulletin, **21**, 197 (1957); **22**, 91, 98, 238 (1958).
33) L.E. Rhuland., *J. Bact.*, **73**, 778 (1957).
34) P. Meadow et al., *Biochem. J.*, **66**, 270 (1957).

Betaine is known as an intermediate metabolite of choline, which degrades through the path of betaine, dimethyl-glycine, sarcosine, reaching to glycine. The liberated methyl group are reported to make transmethylation reaction with various substances like homocysteine[35]. However, the substances relating the path were all ineffective to *Ped. soyae* except betaine (Table III).

On the nutritional effects of amines, Herbst and Snell[36,37] first reported that spermine, spermidine, putrescine or agmatine were essential growth factors for *Haemophilus parainfluenzae*. Momiyama and Miyaki[38] reported that putrescine, agmatine or arcaine were required by *Escherichia*, *Aerobactor* and *Salmonella*. That ethanolamine was effective for stimulating the growth of *Proteus morganii* was also reported by the same authors[39]. Concerning the lactic acid bacteria, it was observed by Kihara and Snell[40] that the growth of *Lactobacillus casei* was markedly accelerated by the co-operation of strepogenin and spermine.

As for the nutrition of *Ped. soyae*, choline is quite ineffective because it is included as a constituent in the basal synthetic medium. Other 22 amines listed in Table IV were also not effective in inducing the growth of *Ped. soyae* when tested at the concentration of 10 mg/l.

V S-factors effects represented by betaine.

The fact that the supplementation of basal medium with betaine only resulted in the half-maximal growth offers an another problem whether the growth stimulating effect of P- or S-factor could be wholly attributed to that of betaine. In order to examine this, experiments were conducted as follows. *Ped. soyae* was grown in both 18% salted and unsalted media supplemented with P-factor (Polypepton) only, with P-factor and betaine, with P-factor and S-factor (yeast extract), and with betaine only. Results given in Fig. 3 shows that maximal growth

Table III. Effect of Betaine and its Related Substances

	O.D.	N/20 NaOH ml
None	0.13	2.14
Betaine HCl 20mg/l	0.31	4.20
Lecithine 100mg/l	0.17	2.15
Acetyl choline chloride 20mg/l	0.14	2.30
Sarcosine 20mg/l	0.13	2.05
Procaine HCl 20mg/l	0.13	2.00
Putrescine 20mg/l	0.12	2.26
Dimethyl-glycine HCl 100mg/l	0.12	2.23
// 10mg/l	0.13	2.15
// 1mg/l	0.13	2.00
Dimethyl-glycine-methyl ester 100mg/l	0.13	2.18
// 10mg/l	0.12	2.05
// 1mg/l	0.13	2.22

After 17 days of incubation, the cell growth was assayed turbidimetrically and acidimetrically.

Table IV. Effect of Various Amines

	4 days	9 days
None	0.01	0.14
Betaine	0.45	0.47
Trimethylamine	0.01	0.15
Monoethylamine	0.01	0.14
Diethyl amine	0.01	0.15
Triethyl amine	0.01	0.15
Monoethanol amine	0.01	0.16
Diethylamino ethanol	0.02	0.14
Tetramethylammonium hydroxyde	0.02	0.06
β-phenyl-ethylamine	0.01	0.14
Dicyclohexyl amine	0.01	0.14
Tyramine HCl	0.01	0.15
Putrescine 2HCl	0.01	0.14
Cadaverine 2HCl	0.01	0.15
Histamine 2HCl	0.00	0.15
DL-Ornithine HCl	0.00	0.14
Agmatine H_2SO_4	0.01	0.15
Tryptamine	0.01	0.15
Isoamylamine	0.00	0.15
Spermine	0.01	0.14
Spermidine	0.00	0.14
Ergothioneine	0.00	0.13
Creatine[41,42]	0.00	0.14
Creatinine	0.00	0.14
Mixture of all	0.00	0.00

The table shows the cell growth by optical density.

35) D.M. Greenberg, Chemical Pathways of Metabolism, vol. **2**, 117 (1954).
36) E.J. Herbst and E.E. Snell, *J. Biol. Chem.*, **176**, 989 (1948); **181**, 47 (1949).
37) E.J. Herbst, E.B. Glinos and L.H. Amunndsen, *Federation Proc.*, **11**, 229 (1952); *J. Biol. Chem.*, **141**, 47 (1955).
38) H. Momiyama, *Nippon Eiseigaku Zasshi*, **10**, 42 (1956); **11**, 291 (1957).
39) H. Momiyama, *ibid.*, **11**, 296 (1957).
40) H. Kihara and E.E. Snell, *Proc. Natl. Acad. Sci.* **43**, 867 (1957).

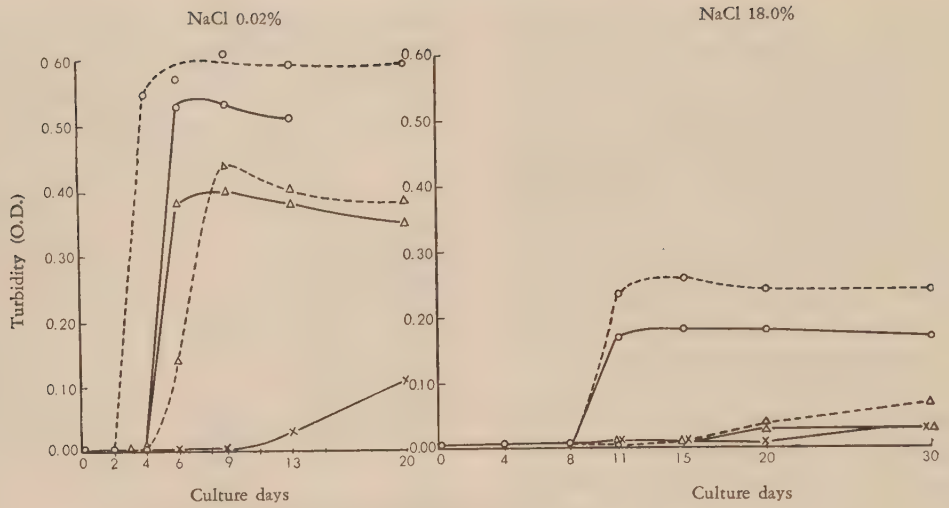

FIG. 3. S-factor Effect Represented by Betaine.

—×— Synthetic medium
—△— +Polypepton 1.0%
—○— +Polypepton 1.0% & yeast extract 0.3%
---△--- +betaine 30 mg/l
---○--- +betaine 30 mg/l Polypepton 1.0%

response was obtained by the addition of both P-factor and betaine as well as in the case of P-factor plus S-factor under the both environments of salted and unsalted media. Thus the effect of betaine is identical with that of S-factor. It was also noted that the single supplement with either betaine or P-factor to 18% salted media showed almost null growth even after thirty days of incubation.

VI. Existence of betaine in yeast extract and beef extract.

As S-factor rich substances, yeast extract, beef extract, heart infusion broth (Difco)[3], unpasteurized soy sauce, and water extract of *Aspergillus sojae* mycelium[1] have been described in former reports. Sumi[43] reported the existence of a considerable amount (0.9 g/100 g) of betaine in spores of *Aspergillus*. Udō[44] in his laborious work on the constituents of soy sauce also found betaine with its sweet taste. Although betaine is widely distributed in plants[45], no information has been reported on its presence in yeast extract and beef extract. It is also questionable that the growth promoting substance in yeast extract or beef extract is only betaine.

The experiments were conducted chromatographically to identify the biologically active spot and the spot of betaine on paper. Yeast extract and beef extract (Difco) were extracted with ethanol, the soluble fraction and the residue were each spotted on the lower tips of paper strips (Tōyō Roshi No. 53) and developped for about thirty hours at 20°C using the solvent of *n*-butanol, acetic acid and water mixed in the ratio of 4 : 1 : 2. To detect the microbiologically active spot, the developped and dried paper strip was cut in each 1 cm, put into cotton plugged test tubes, poured by the 5 ml of basal synthetic medium, and their growth promoting acitivites were assayed as usual. The colour spot of tertiary amines and tetrary ammonium salts including choline and betaine was detected by spraying Dragendorff's reagent. The samples added with betaine were also chromatographed to calibrate the R_F of betaine in crude mixtures.

41) R.H. Nimmo-Smith and G. Appleyard, *J. Gen. Microbiol.*, **14**, 336 (1956).
42) G. Appleyard and D.D. Woods, *ibid.*, **14**, 351 (1957).
43) M. Sumi, *J. Agr. Chem. Soc. Japan*, **2**, 866 (1925).
44) S. Udo, *ibid.*, **7**, 852 (1931); **8**, 675 (1932).
45) M. Guggenheim, Die Biogenenaminen, p. 193 (1940).

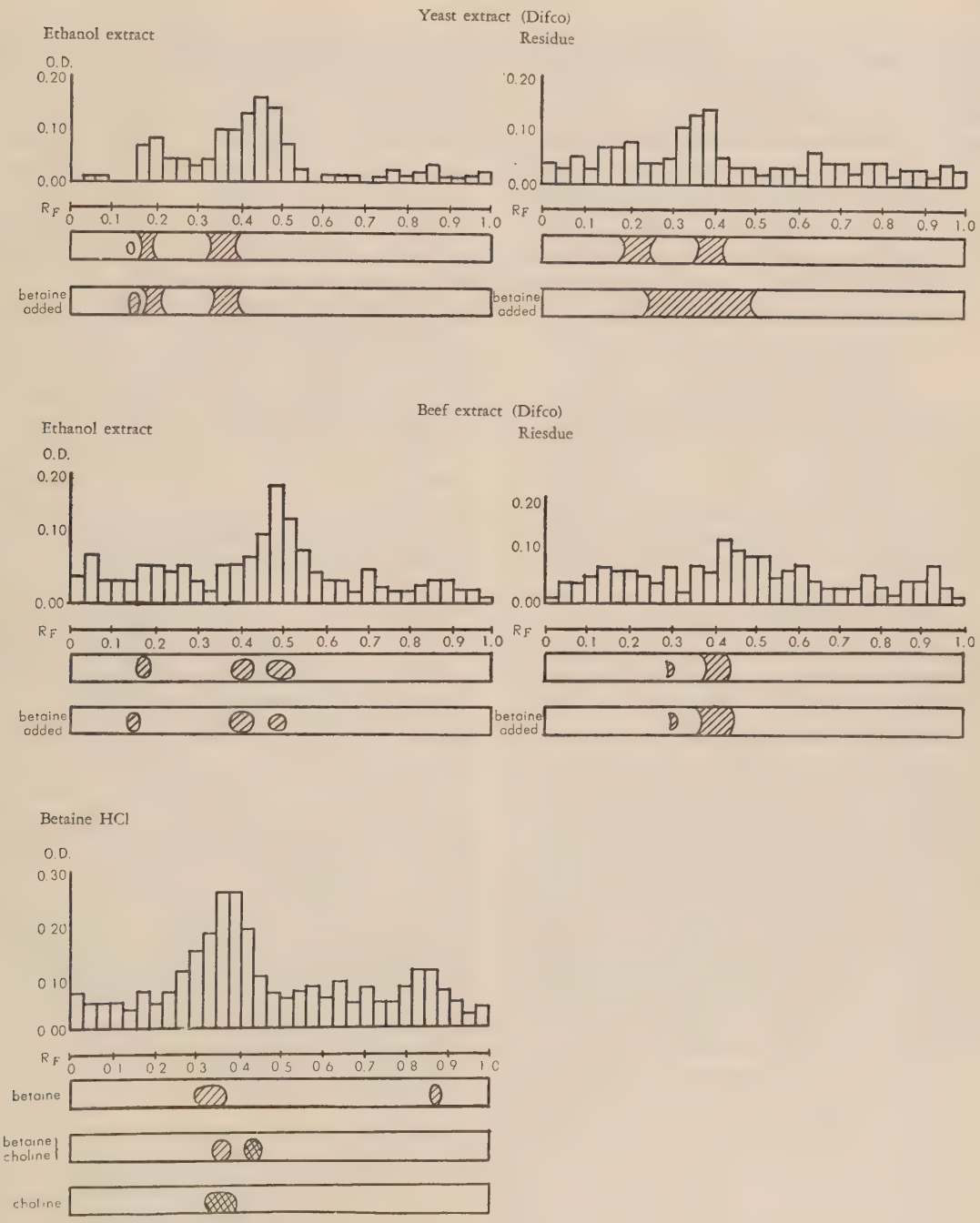

FIG. 4. Bioautograms of Yeast Extract, Beef Extract and Betaine-HCl, and their Paper Chromatograms Sprayed by Dragendorff's Reagent.

Fig. 4 shows that the growth stimulants are rather ethanol soluble, Dragendorff positive spots and the spot of mixed betaine are included in the area of biologically active spots. The active spots which do not coincide with the spots of tertiary amines may be partly due to the action of contaminating P-factor. Such phenomena is rather distinct in the case of yeast extract. In general, at least a part of the S-factor substances in yeast extract or beef extract are attributable to betaine contained in them.

DISCUSSION

Pediococcus soyae is a very specific lactic acid bacterium in the respect of its high osmotolerant character growing even in 24~26% salted media[1]. This organism grows only very poorly in the ordinary synthetic medium employed for bioassay[5], and requires two different specific growth factors for maximal growth. One of them, provisionally named as S-factor in former reports, is a constituent of Difco's yeast extract, beef extract, heart infusion broth, unpasteurized Japanese soy sauce, etc.. In this report it is elucidated that the action of S-factor was perfectly replaced by the addition of betaine hydrochloride 30 mg/l. It was therefore observed that at least one of the active constituents in the yeast extract or beef extract was betaine. However, the problem that some other growth stimulating substances might exist in them is still remained to be clarified. The optimum concentration of betaine hydrochloride as high as 30 mg/l also sustains this question because it is too high as compared with the required amounts of ordinary vitamins and it almost nears to the level of amino acids or other substrates.

Betaine is a usual component in plants[45], and it is expected that the substance may play some roles in their metabolism and physiology. It has been known that betaine is an intermetabolite of choline, and it reacts as a substrate of transmethylation. It has an effect as a lipotropic factor for the prevention of animal fat liver, and also as a growth factor substitutable with choline. However, betaine is useless, in contrast to choline, for the prevention of chick's perosis and for its growth stimulation[46,47]. As for the effects of betaine against *Ped. soyae*, the growth promoting effect and the osmotolerancy bestowing effect, are the unique faculty of betaine because no other metabolically related substances such as choline, dimethyl glycine, sarcosine or methionine are effective. It is supposed from these facts that betaine is effective for *Ped. soyae* not through the metabolic path ever known, but may be directly effective for maintaining the semi-permeability of the cell membrane in co-operation with P-factor.

The author wishes to express his hearty thanks to Prof. T. Asai and Prof. K. Kitahara of the Institute of Applied Microbiology, University of Tokyo for their kind guidances. He is greatly indebted to Prof. K. Miyaki and Assist. Prof. M. Hayashi of Chiba University for their kind advices and gift of dimethylglycine. Generous supply of many precious vitamins and growth factors were made by Assist. Prof. G. Tamura and Mr. T. Fukami of University of Tokyo. The author's thanks are also due to the kind advices given by Dr. Mogi and members of this institute. He is especially grateful to Mr. T. Kuramochi for his earnest and able assistance.

46) A.D. Welch, *J. Nutr.*, **40**, 113 (1950).
47) T.H. Jukes and A.D. Welch, *J. Biol. Chem.*, **146**, 19 (1942).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 497~500, 1960]

On the Mechanism of Metabolism of (−)-Epicatechin

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(−)-Epicatechin was administered orally to rabbits and vanillic acid, 3-hydroxybenzoic acid, protocatechuic acid, and three kinds of neutral substances were found to be excreted in the urine. The three kinds of neutral substances were identified as 1-δ-(3-methoxy-4-hydroxyphenyl)-, 1-δ-(3-hydroxyphenyl)-, and 1-δ-(3,4-dihydroxyphenyl)-γ-valerolactones, which are optical isomers of the three kinds of neutral substances excreted after administration of (+)-catechin. From the presence of these intermediate metabolites, it was verified that (−)-epicatechin is metabolized by the same mechanism as (+)-catechin described earlier.

In this laboratory, (+)-catechin was administered orally to rabbits, the urine was collected, and three kinds of acid substances and three kinds of neutral substances (F, G, and H) were found to be excreted in the urine as intermediate metabolic products of (+)-catechin. The acid substances were found to be vanillic, 3-hydroxybenzoic, and protocatechuic acids[1], and the neutral substances were recognized as new compounds not described in past literature[2]. Structural study of these compounds revealed that F is δ-(3-methoxy-4-hydroxy-phenyl)-, G is δ-(3-hydroxyphenyl)-, and H is δ-(3,4-dihydroxyphenyl)-γ-valerolactone[3~6]. By deduction from these six kinds of intermediate metabolites, the metabolic route of (+)-catechin was established[7].

(−)-Epicatechin also occurs in nature and is contained in comparatively large amount in tea leaves and cacao. Therefore, it would be of interest to find how (−)-epicatechin, a stereoisomer of (+)-catechin, is metabolized in the animal body. While (+)-catechin is contained in great quantity in Gambia catechu and is easily isolated (−)-epicatechin is present as a mixture with other catechins such as (+)-catechin, (−)-epigallocatechin, (+)-gallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate, and its pure isolation is extremely difficult. (−)-Epicatechin was finally isolated in a resonable quantity and in comparative purity being contaminated to some extent by (−)-epigallocatechin, from tea leaves. This (−)-epicatechin was administered orally to rabbits, same as was the (+)-catechin, and an extract from their urine was examined by paper partition chromatography. Both the acidic and neutral portions produced approximately the same chromatogram as did the (+)-catechin except that a new spot (X) appeared in the neutral portion, which was not found in the case of the latter.

Further, isolation of the neutral substances was attempted by column chromatography through cellulose powder. Due to the small amount of the sample available, substances corresponding to F and G from (+)-catechin could not be isolated as crystals, but the substance (H′) corresponding to H was isolated in crystalline state. Detailed examination of the chemicophysical

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1) Y. Oshima, H. Watanabe and S. Isakari, *J. Biochem. (Japan)*, **45**, 861 (1958).
2) Y. Oshima and H. Watanabe, *ibid.*, **45**, 973 (1958).
3) H. Watanabe, This Bulletin, **23**, 257 (1959).
4) H. Watanabe, *ibid.*, **23**, 260 (1959).
5) H. Watanabe, *ibid.*, **23**, 263 (1959).
6) H. Watanabe, *ibid.*, **23**, 268 (1959).
7) H. Watanabe, *ibid.*, **23**, 268 (1959).

properties of this substance revealed that it has the same chemical strucutre but is an optical isomer of H. Since the substances F′ and G′ corresponding to F and G give the same color reaction and R_F values as those of F and G, and considering the relationship between H and H′, it is assumed that F′ and G′ are likewise optical isomers of F and G. This has made it clear that the mechanism of metabolism of (−)-epicatechin is the same as that of (+)-catechin.

EXPERIMENT AND RESULTS

1. Extraction, Isolation, and Purification of (−)-Epicatechin

While examining the product through paper chromatography, isolation and purification were carried out based on the method presently used to isolate tea catechin[8].

About 8 kg of domestic green tea was extracted four times with hot water; aqueous solution of lead acetate was added to the extract solution, and the resulting prescipitate was filtered off. Ammonium hydroxide solution was added to the filtrate, the newly formed precipitate was collected, decomposed with sulfuric acid and hydrogen sulfide, and filtered. The filtrate was extracted three times with ethyl acetate. The ethyl acetate solution was dried, evaporated, and poured into dehydrated chloroform. The precipitate formed at this stage contained, (−)-epicatechin, gallocatechin and catechin gallate. Recrystallization of this precipitate from water resulted in the initial precipitation of (−)-epicatechin which was collected and this recrystallization, repeated several times, finally yielded a crystalline precipitate. This recrystallization was repeated further and about 20 g of (−)-epicatechin crystals of comparatively high purity were obtained. Examination of this substance by paper chromatography revealed, as shown in Fig. 1, that it still contained a minute amount of gallocatechin and catechin gallate.

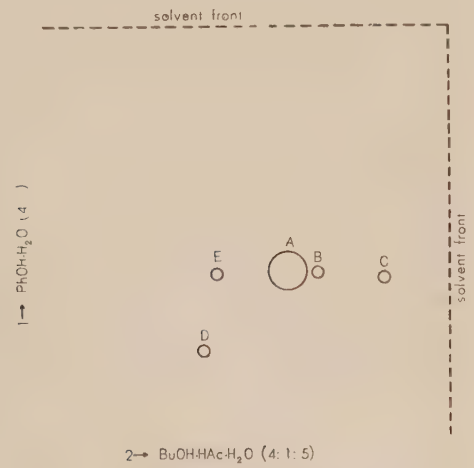

FIG. 1. Paper Partition Chromatogram of (−)-Epicatechin extracted from Green Tea

A: (−)-Epicatechin
B: (+)-Catechin
C: (−)-Epicatechin gallate
D: (−)-Epigallocatechin
E: Rutin

8) Y. Oshima, *Nippon Nogei-kagaku Kaishi*, **12**, 1 (1936); **9**, 448 (1933).

2. Collection of Urine

Two rabbits each weighting 2 kg were fed "Okara" (residue of soya bean powder extracted by hot water) containing 1.2 g of (−)-epicatechin per head per day for five days. The urine was collected (total, 1.31) as in the case of (+)-catechin reported in the preceding paper[1].

3. Extraction of Intermediate Metabolites

The urine (1.31) was saturated with sodium chloride and acid and neutral intermediate metabolites were extracted three times with equal quantities of ethyl acetate. The acetate extract was dried over anhydrous sodium sulfate and concentrated to about 100 cc. In order to separate neutral substances, the concentrated ethyl acetate extract was shaken with 20 cc of ammonium carbonate soluion to locate the acid substances in aqueous layer. The aqueous layer was saturated with sodium chloride and extracted with two 50 cc portions of ethyl acetate. The combined acetate layer was dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated, leaving 0.2 g of an oily neutral substances. In order to separate acid substances the aqueous layer was acidified with hydrochloric acid and extracted with three 50 cc portions of ethyl acetate. The acetate layer was dried, filtered, and evaporated, leaving 0.2 g of an oily acid substances. As a control, the same experiment was carried out with the urine from untreated rabbits.

4. Paper Partition Chromatography of Acid Sub-

TABLE I. COLOR REACTIONS AND R_F VALUES OF ACID SUBSTANCES FROM THE URINE OF RABBITS ADMINISTERED (−)-EPICATECHIN.

Spots	Color Reactions	R_F Values
A′	Orange	0.79
C′	Yellow	0.23
E′	Red then black	0.02

stances

Chromatography was carried out with Toyo Roshi No. 50 filter paper, developed with water-saturated benzene and acetic acid mixture (9 : 1 by volume) as a solvent, and colored by the method of Bray and others[9]. Comparison with the chromatogram of the control revealed three new spots (A′, C′ and E′), whose R_F values and color reactions are presented in Table I.

There was no marked difference, other than that reported above, from the result obtained with (+)-catechin.

5. Paper Partition Chromatography of Neutral Substances

Paper partition chromatography was carried out exactly the same as in the case of acid substance. While no spot was detected from the control urine, four new spots (F′, G′, H′, and X) were detected in the urine of rabbits administered (−)-epicatechin. The R_F values and color reactions of these spots given in Table II.

Table II. Color Reactions and R_F Values of Neutral Substances from the Urine of Rabbits Administered (−)-Epicatechin.

Spots	Color Reactions	R_F Values
F′	Pink	0.93
G′	Yellow	0.71
X	Orange-yellow	0.39
H′	Red-purple then black-brown	0.12

6. Column Chromatography of Neutral Substances

From the result of paper partition chromatography of the neutral substances described above, the spots F′, G′ and H′ were assumed to have the same structure as the neutral substances F, G, and H, obtained after administration of (+)-catechin. In order to further verify this point and to clarify the nature of the spot X, separation of the neutral substance was attempted through column chromatography.

A solution of 0.2 g of the oily neutral substance, obtained by extraction, dissolved in 0.5 cc of ethyl acetate was poured into a column (internal diameter, 2.8 cm, length, 30 cm) of cellulose powder (The column was developed with a solvent system of benzene-acetic acid-water (*loc. cit.*), and collected in 10 cc fractions. One drop of each fraction was spotted on a filter paper and examined by Bray's coloration method. Fractions giving color reactions for F′, G′, and X were each collected and the solvent was distilled off under reduced pressure. All of these fraction left only a trace of the substance, and none of the products could be isolated in crystalline state. The substance H′ was hardly eluted by this solvent system and the column was eluted with ethyl acetate. Fractions giving color reaction corresponding to H′ were collected; the solvent was distilled off, and the residue crystallized gradually on cooling. The residue was washed with 10 cc of ether to remove insoluble matter and crude crystals thereby formed were repeatedly recrystallized from water, yielding ca. 50 mg colorless, prismatic crystals.

9) H.G. Bray, W.V. Thorpe and K. White, *Biochem. J.*, **46**, 271 (1950).

7. Determination of Chemical Structure of the Neutral Substance (H′)

(a) Determination of Infrared Absorption Spectrum - The infrared absorption spectrum of the neutral substance, shown in Fig. 2 (*l*), was identical with that (*d*) of the substance H obtained by administration of (+)-catechin and, therefore, H′ was assumed to have the same structure.

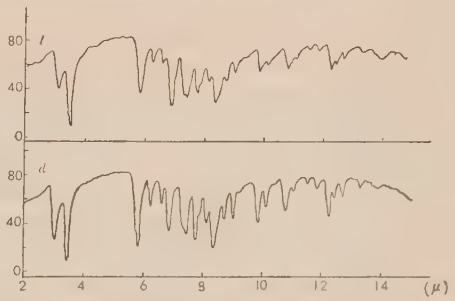

Fig. 2. Infrared Absorption Spectra of *d*-δ-(3,4-Dihydroxyphenyl)-γ-valerolactone (*d*) and H′ (*l*).

(b) Melting point - The meltiog point, 145∼146°, of H′ agreed with that of H obtained previously. Therefore, equal amounts of H and H′ were mixed, fused, and the melting point of the solidified mixture was measured. This mixture melted in a lower range (132∼133°) and the melting point agreed with that of *dl*-δ-(3,4-dihydroxyphenyl)-γ-valerolactone, synthesized earlier. Their admixture showed no depression of the melting point (m. p. 132∼133°), and the two were found to be identical.

(c) Optical Rotation - Optical rotation was measured in dehydrated ethanol at 20°, and the value was $[\alpha]_D^{20}$ −24.8°.

DISCUSSION

Oral administration of (−)-epicatechin to rabbits showed that three kinds of new substances, A′, C′, and E′, are excreted in the urine. These

substances were assumed, to be identical with substances A, C, and E excreted in urine after administration of (+)-catechin, from the result of R_F values and color reaction in paper chromatography*. Substances corresponding to B and D from (+)-catecin administration were not detected. These substances were obtained in trace amounts after administration of (+)-catechin and their absence must be due to slight differences in extraction and other conditions.

Four new constituents F′, G′, H′, and X were detected as the neutral substance by paper partition chromatography. It was assumed from their R_F values and color reactions that F′, G′, and H′ have the same structure as F, G, and H. Since (+)-catechin and (−)-epicatechin have different optical properties and since F, G, and H have asymmetric carbon atom in each molecule, there were still doubts about optical properties. Therefore, the neutral substances were submitted to column chromatography and H′ was isolated in crystalline state. The infrared absorption spectrum of H′ was in agreement with that of H and the two were found to have identical structure. Further, H and H′ showed the same melting point and equal mixture of these two substances showed the same melting point as that of the racemic compound obtained by synthesis. Further, admixture with the racemic compound showed no depression of the melting point. Since the measurement of optical rotation of H in dehydrated ethanol showed that it is dextrorotatory, H′ was thought to be levorotatory and this was bourne out by its optical rotation of $[\alpha]_D^{20}$ −24.8°, as against $[\alpha]_D^{16}$ +25.7° of H from (+)-catechin. This result, together with infrared absorption spectral data and melting point measurements proved H and H′ to be optical isomers.

Due to the samall amount available, F′ and G′ could not be isolated in crystalline state, but it was assumed from the R_F values and color reactions that they have structures identical with F and G, and are probably optical isomers, as was the case with H and H′. It is therefore assumed that F′, G′, and H′ are respectively l-δ-(3-methoxy-4-hydroxyphenyl)-, l-δ-(3-hydroxyphenyl)- and l-δ-(3,4-dihydroxyphenyl)-γ-valerolactones.

Based on the formation of these six kinds of neutral and acid inteamediate metabolites, the mechanism of metabolism of (−)-epicatechin is entirely identical with that of (+)-catechin[7].

Difference in the optical properties of neutral substances is natural from the optical difference of (+)-catechin and (−)-epicatechin.

Identification of X was not possible by paper partition or column chromatography.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 501~505, 1960]

Biochemical Studies on the Formation of the Silkprotein

X. Ordard Progression of Fibroin in the Inside of the Silkgland during the Fifth Instar (Continuation)

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Received December 24, 1959

The progression of fibroin in the inside of the silkgland during growth of the fifth instar was elucidated by tracing radioactive fibroin which was made of C^{14} glycine. Each fibroin synthesized by the posterior division at different period of the fifth instar moves, in an ordered fashion, in the inside of the gland along the middle and anterior division during growth of the silkworm. Furthermore, fibroin present in the middle division of the silkworm just before spinning is due to the successive disposition, in the order synthesized, of each portion of fibroin synthesized at different periods of the fifth stage.

Biosynthesis of the silkprotein in a silkworm larva takes place in a pair of the silkgland chiefly at the fifth stage; the fibroin in cells of the posterior division and the sericin in cells of the middle division. The liquid fibroin excreted from cells of the posterior division is transferred to the middle divisions. During the time when liquid fibroin is stored in the middle division, it is gelled and its surface is covered with the sericin which has been excreted from there. At maturity**, fibroin and sericin present in the middle division are spun as a pair of threads out of the body via the anterior division and the spinneret.

Many studies have been chiefly carried out, from the biological standpoint, on the excretion of fibroin and sericin in cells of the silkgland. However, little is known of the progression of fibroin in the inside of the silkgland except a few reports. Ogiwara[1] clarified the distribution of fibroin in the reservoir of the silkgland by dyeing, with Delafields' haematoxylin, the silkgland of the silkworm at the different period of the fifth instar. Nakagawa, one of the authors, tried recently to elucidate the progression of fibroin in the inside of the silkgland during growth of the silkworm by tracing the fibroin dyed with a pigment "thionin" which was given to silkworms. Fukuda and Florkin[2] investigated, using the European race of silkworm, the progression of the fibroin in the reservoir of the silkgland by tracing, to the matured larva, the radioactive fibroin which was made of the glycine labeled with C^{14} at the sixth day of the fifth instar, and found that the fibroin synthesized by the posterior division moves, in an ordered fashion, in the inside of the gland along the middle and the anterior division during growth of the silkworm.

The current experiment is performed to elucidate how each fibroin synthesized by the posterior division at different periods of the fifth instar moves in the inside of the silkgland during growth of the silkworm.

EXPERIMENTAL

1) Administration of C^{14} glycine to silkworms

* The Rakuto High School, Kyoto.

** "Maturity" corresponds to the moment when silkworms stop eating mulberry leaves at the end of the fifth instar, i.e., about one day before spinning.

1) S. Ogiwara, *J. Sericul. Sci. Japan*, **11**, 55 (1940).

2) T. Fukuda and M. Florkin, *Arch. internet. Physiol. Biochem.*, **67**, 214 (1959).

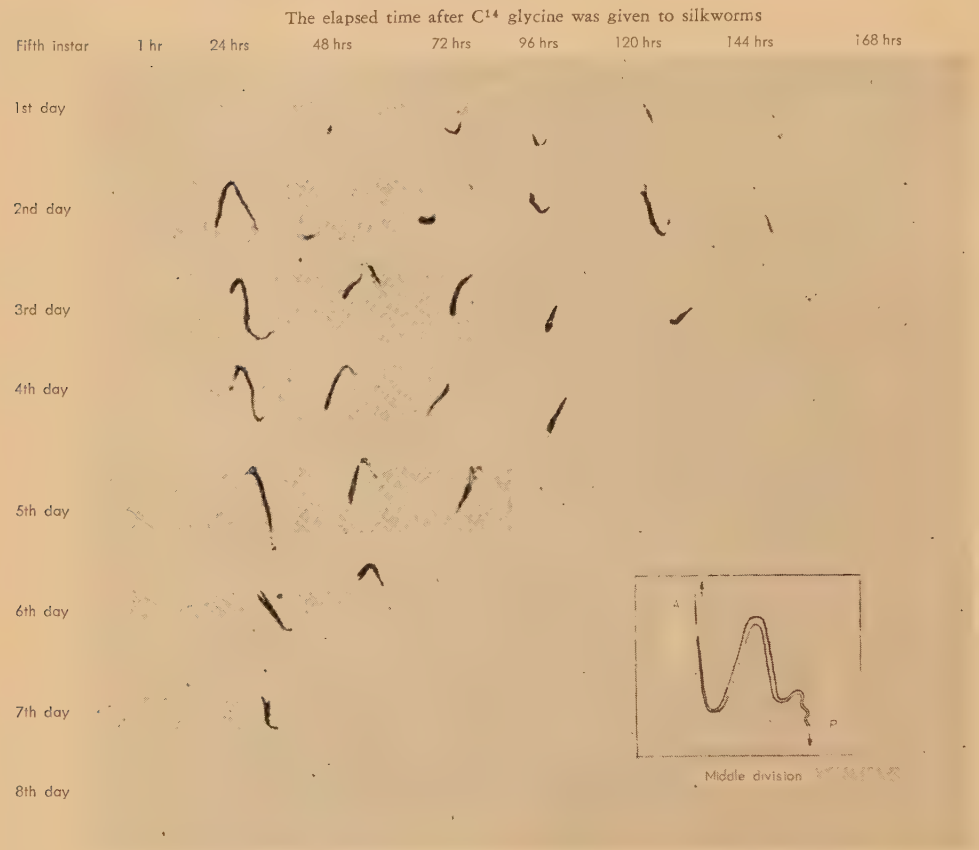

FIG. 1. Radioautographs of the Longitudinal Section of the Middle Division of the Silkgland
A: Anterior division. P: Posterior division.

Glycine-1-C^{14} (4.1 mc/mM) was obtained from the Radiochemical Centre, Amersham, England. The radioactive glycine 0.5 μc per larva was given per os to the silkworms, Si 122×Nichi 122, in different periods from the beginning of the fifth instar to the end of the fifth instar. Reared on mulberry leaves, the worms were successively dissected in 0.8 per cent solution of sodium chloride at intervals of 24 hours after the radioactive meal, and a pair of silkglands isolated.

2) Radioautograph of the longitudinal section of the middle division of the silkgland

After the silkglands were washed in 0.8 per cent solution of sodium chloride, the middle division of one of the glands was frozen in dry ice, and a longitudinal section, about 0.5 mm thick, was prepared, using a razor blade. The slice was degummed by simmering for two hours in a M/50 solution of sodium carbonate, and dried. By superposing, for three weeks, an X-ray film on each slice, radioautographs of the different slices were obtained (Fig. 1). Among the silkworms, given radioactive glycine, those that elapsed one hour and 24 hours from the first day of the fifth instar, and that elapsed 24 hours from the second day of the fifth instar were not used for making radioautographs of the longitudinal section of the middle division of the silkgland, because the fibroin present in the inside of the middle division of these silkworms was in extremely small amount.

RESULTS

Radioautographs of the longitudinal section of the middle division of the silkgland are shown in Fig. 1.

In such a case that C^{14} glycine was given to the silkworm at the second day of the fifth

instar, the radioactive fibroin synthesized by the posterior division using the C^{14} glycine is found in a wide extent from the middle part of the middle division to the posterior part of the middle division at the 24 th hour after the radioactive meal, in the anterior one-third of the middle part at the 48 th hour, in an extent from the anterior one-third of the middle part to the posterior one-third of the anterior part at the 72 nd hour, and in the anterior part from the 96 th hour to the 144 hour (reaching maturity). In such a case when C^{14} glycine was given to the silkworm at the fourth day of the fifth instar, the radioactive fibroin derived from the posterior division is found in all the posterior part of the middle division at the 24 th hour, in the posterior one-third of the middle part at the 48 th hour, in the middle of the middle part at the 72 nd hour, in the anterior one-third of the middle part at the 96 th hour (reaching maturity). When C^{14} glycine was given to the silkworm at the sixth day of the fifth instar, the radioactive fibroin derived from the posterior division is found in the posterior one-third of the posterior part of the middle division at the 24 th hour and in an extent from the posterior one-third of the middle part to the anterior one-third of the posterior part at the 48 th hour (reaching maturity).

This radioautograph shows that the C^{14} glycine given to the silkworm is absorbed in abundance from all the cells of the posterior divison, but some C^{14} glycine, in extremely small amount, also from a fixed position of the middle division.

At one hour after the radioactive meal, the radioactive fibroin synthesized using the C^{14} glycine by the cells of the posterior division is still located in the posterior division, in common to all the silkworm, not reaching until the inside of the middle division (Fig. 2.). At the 24 th hour after the radioactive meal, such a tendency, that the radioactive fibroin derived from the posterior division distributes extensively in the inside of the middle division, is recognized. This tendency is especially remarkable at the silkworms at the beginning of the fifth instar whose middle division is not filled yet with enough fibroin.

After the lapse of 48 hours, the distribution of the radioactive fibroin in the inside of the middle division becomes confined, in common to all the silkworms, with narrow limits.

DISCUSSION

A method dyeing the silkgland with Delafields' haematoxylin, devised by Ogiwara[1], for observing the progression of fibroin in the reservoir of the silkgland is a good way for finding the location of fibroin present in the inside of the middle division. It is, however, impossible by this method to trace the progression of the fibroin itself, which was synthesized by the posterior division in a fixed period, in the inside of the middle division during growth of the silkworm. A method dyeing fibroin in the silkgland with a pigment "thionin" which dyes fibroin, but not sericin, devised by

FIG. 2. Radioautograph of a whole silkgland of the silkworm that consumed C^{14} glycine at the fourth day of the fifth instar.
One hour after the radioactive meal

Nakagawa, one of the authors, makes it possible to seize certainly the progression of fibroin in the inside of the middle division during the growth of the silkworm, but has a weak point as follows. Thionin given to the silkworm body transfers in abundance into the posterior division, but some thionin is also absorbed from a fixed position of the middle division. In such a case that thionin is given to the silkworm whose middle division has already had a great amount of fibroin inside, the fibroin present in the middle division ought to be dyed directly with thionin which came through cells of the middle division. Consequently, it becomes somewhat difficult to trace only the fibroin derived from the posterior division in the inside of the middle division. In fact, in a comparative experiment on the progression of fibroin in the silkgland, carried out with the both, thionin and C^{14} glycine, such a tendency, that the fibroin dyed with thionin distributes more wide than the fibroin labeled with C^{14} in the inside of the middle division, is recognized. In observation of the behavior of fibroin in the reservoir of the silkgland, it seems to be beyond question that the fibroin labeled with C^{14} is more desirable, as a tracer, than the fibroin dyed with thionin. As one of the main constituent amino acids of fibroin is glycine (42.8 per cent), C^{14} glycine was especially adopted for this study to produce radioactive fibroin in the silkworm body. C^{14} glycine given to the silkworm transfers in abundance to all the cells of the posterior division and is used for formation of fibroin there, but small amount of C^{14} glycine is also absorbed, as shown in Fig. 2, from a fixed position of the middle division to produce sericin. Furthermore, when C^{14} glycine was given to the silkworm, a small amount of C^{14} glycine is also used for growth of the silkgland-cell itself during the fifth stage. In making a radioautograph of the longitudinal section of the middle division, a careful consideration was, therefore, given to remove radioactive sericin and silkgland-cell as perfect as possible from the middle division.

As shown in Fig. 1, each fibroin synthesized by the posterior division at different periods of the fifth instar seems to move, in an ordered fashion, in the inside of the gland along the middle and the anterior division during growth of the silkworm. Fig. 3, showing accumulation of each fibroin, which has been synthesized by the posterior division at different periods of the fifth instar, in the middle division of the silkworm at the end of the fifth stage seems to suggest that the fibroin present in the inside of the middle division at the end of the fifth stage is due to successive disposition, in the order synthesized, of each portion of fibroin synthesized at different periods of the fifth stage.

As shown in Fig. 1, radioautographs of the longitudinal section of the middle division, the radioactive fibroin is not recognized, in common to all the silkworms, in the middle division of the silkgland at one hour after the radioactive meal. This phenomenon is due to a fact that

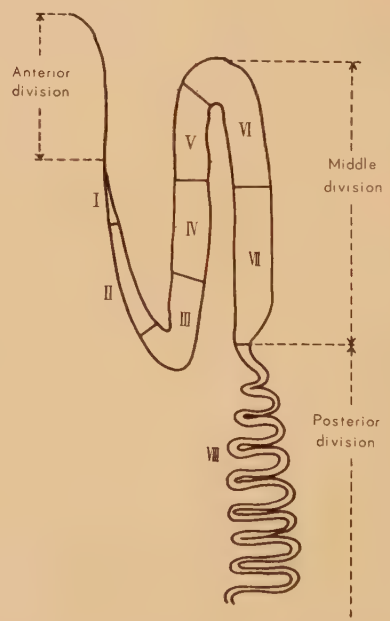

FIG. 3. Location of Each Fibroin, Which was Synthesized by the Posterior Division at Different Periods of the Fifth Instar, in the Middle Division of the Silkworm at the End of the Fifth Stage.

Roman numerals (I~VIII) indicate the days of the fifth instar on which the radioactive fibroin was synthesized using the C^{14} glycine.

the transfer of the fibroin synthesized by the posterior division to the middle division does not yet take place within one hour (Fig. 2). At the 24th hour after the radioactive meal, the radioactive fibroin distributes in a comparatively wide extent, in the inside of the middle division. Especially, this tendency is remarkable at the silkworm at the beginning of the fifth instar whose middle division has not yet enough fibroin inside. This phenomenon will be explained by reasons as follows.

In a case when fibroin is synthesized in all the cells of the posterior division which is comparatively long, the fibroin synthesized by cells of the posterior division near the middle division transfers naturally to the middle division more rapid than the fibroin synthesized at the end of the posterior division.

Also, the fibroin synthesized by the posterior division at the beginning of the fifth instar whose middle division has not yet enough fibroin inside can move comparatively rapid, and distribute in a comparatively wide extent in the middle division.

SUMMARY

1) The current experiment was performed to clarify the progression of fibroin in the inside of the silkgland during growth of the fifth instar, by tracing radioactive fibroin which was made of C^{14} glycine.

2) The radioactive glycine, 0.5μc per larva, was given per os to silkworms, Si 122×Nichi 122, in different periods from the beginning of the fifth instar to the end of the fifth instar. These worms were reared on mulberry leaves until they became matured larvae, one day before spinning. At intervals of 24 hours after the radioactive meal, the silkgland isolated from silkworm was used for making a radioautograph of the longitudinal section of the middle division.

3) Each fibroin synthesized by the posterior division at different period of the fifth instar moves in an ordered fashion as shown in Fig. 1, in the inside of the gland along the middle and the anterior division during growth of the silkworm.

4) Fibroin present in the middle division at the end of the fifth stage is due to the successive disposition, in the order synthesized, of each portion of fibroin synthesized at different periods of the fifth stage.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 506~508, 1960]

Studies on the Reduction of Terpenes with Sodium in Aqueous Ammonia

Part VI. Syntheses of (±)-Menthol Isomers*

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Received December 29, 1959

All isomers of (±)-menthol were prepared. Particularly, (±)-menthol and (±)-isomenthol were obtained in a good yield from (±)-menthone and (±)-isomenthone respectively, by the treatment with sodium in aqueous ammonia, i.e., a novel method of reduction employed in this series. Besides, it was found that the substance, m.p. 121°, reported for (±)-menthyl 3,5-dinitrobenzoate in the previous literature, is a mixed crystal of (±)-menthyl and (±)-isomenthyl 3,5-dinitrobenzoate.

Read[1] published the synthesis of (±)-menthol involving the reduction of (±)-piperitone with sodium and alcohol. He also prepared (±)-isomenthol by diazo-reaction of (±)-isomenthylamine which was obtained from (±)-piperitone via (±)-isomenthone and (±)-isomenthoneoxime[2]. However, this method was very tedious and resulted in a very poor yield.

In the previous papers, it has been shown that (−)-menthol and (+)-isomenthol were obtained predominantly from (−)-menthone[3] and inverted menthone[4] respectively, by the treatment with sodium in aqueous ammonia, i.e., a novel method of reduction employed in this series.

The present investigation was undertaken to obtain (±)-menthol isomers in a pure state. These isomers were indispensable for the identification of the reduction product of (±)-piperitone and piperitenone.

(±)-Menthol and (±)-isomenthol were prepared in a good yield from (±)-menthone and (±)-isomenthone respectively, by application of the above-mentioned method of reduction. (±)-Neomenthol was prepared from (±)-menthone according to the direction of Read[5]. (±)-Neoisomenthyl 3,5-dinitrobenzoate was prepared from a mixture of (−)-neoisomenthyl and (+)-neoisomenthyl 3,5-dinitrobenzoates in equal amount.

These melting points, except (±)-menthyl 3,5-dinitrobenzoate, agreed well with those reported in the literature[6]. However, the melting point of (±)-menthyl 3,5-dinitrobenzoate (128.5~129.5°) was higher than the value reported (121°). From the observation of infrared spectrum of the regenerated menthol, it was supposed that the substance, m.p. 121°, reported for (±)-menthyl 3,5-dinitrobenzoate, is a mixed crystal of (±)-menthyl and (±)-isomenthyl 3,5-dinitrobenzoate.

EXPERIMENTAL

All melting and boiling points were uncorrected.

* For Part V see This Bulletin, **24**, 402 (1960).

1) J. Read and W.J. Grubb, *J. Chem. Soc.*, **1933**, 170.

2) J. Read, *J. Chem. Soc.*, **1925**, 2784; **1926**, 2231; **1927**, 1279; **1933**, 172.

3) H. Ueda, Y. Shibahara and S. Shimizu, This Bulletin, **23**, 376 (1959).

4) H. Ueda and S. Shimizu, This Bulletin, **23**, 380 (1959).

5) W.J. Grubb and J. Read, *J. Soc. Chem. Ind.*, **53**, 53T (1934).

6) J.L. Simonsen, "The Terpenes", Cambridge Univ. Press, 1953, Vol. I, p. 243; E. Guenther, "The Essential Oils", D. Van Nostrand Company, 1957, Vol. II, p. 222.

Infrared spectra were recorded at Prof. Nakajima's Laboratory, University of Kyoto. Microanalyses were carried out by the Microanalytical Division, Prof. Mitsui's Laboratory, University of Kyoto. Ultra violet absorption spectra were determined at Prof. Shimizu's Laboratory, Shinshu University.

(±)-Menthone

By Beckmann's oxidation of (±)-menthol, m. p. 33~34°, (34.0 g), prepared from thymol by the usual method[7], (±)-menthone (29.0 g, Yield 86.4%) was obtained; b. p. 86~87°/13 mm, d_4^{20} 0.8957, n_D^{20} 1.4506, MR. 46.32 (Calcd. 46.19), $[\alpha]_D^{20}$ ±0° (homog.), semicarbazone m. p. 162~162.5°, oxime m. p. 82~83°, 2,4-dinitrophenylhydrazone m. p. 143.5~144°.

(±)-Menthol and its Derivatives

(±)-Menthone (9.5 g) was reduced with sodium (7.5 g) in aqueous ammonia and benzene according to the procedure described previously[3]. The product was worked up in the usual manner and gave crude (±)-menthol (8.8 g, Yield 91.5%); b. p. 81~83°/6 mm, m. p. 26~34°. The crude (±)-menthol (5.06 g) was converted into crude 3,5-dinintrobenzoate, m. p. 125~128°, (10.75 g, Yield 94.7%). One crystallization from methanol gave pure (±)-menthyl 3,5-dinitrobenzoate, m. p. 128.5~129.5°, (8.51 g, Yield 79.1% from crude ester). The melting point was not alternated by further recrystallization from *n*-hexane. Mixed melting points with (±)-neomentyl 3,5-dinitrobenzoate, m.p. 130~131°, and with (±)-isomenthyl 3,5-dinitrobenzoate, m.p. 129~130°, were 108~115° and 121~122° respectively. *Anal.* Found: C, 58.40; H, 6.42; Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%.

(±)-Menthyl 3,5-dinitrobenzoate (6.00 g) was hydrolysed under reflux with N/2-alcoholic potassium hydroxide. Regenerated (±)-menthol (2.40 g, Yield 89.9%) was obtained as a solid; b. p. 76.0°/5 mm, m. p. 36~37°; *Anal.* Found: C, 76.85; H, 12.76; Calcd. for $C_{10}H_{20}O$: C, 76.86; H, 12.90%. Crystallisation from nitromenthane gave pure (±)-menthol, m. p. 37~38°, and it remained constant after further recrystallization. The infrared spectrum was completely similar to that of (−)-menthol in carbon disulfide solution.

(±)-Menthol gave 3,5-dinitrobenzoate, m. p. 128.5~129.5° (from *n*-hexane), as pale yellow needles (Found: C, 58.26; H, 6.31; Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%); and *p*-nitrobenzoate, m. p. 91~91.5° (from methanol), as pale yellow prisms (Found: C, 67.04; H, 7.63; Calcd. for $C_{17}H_{23}O_4N$: C, 66.86; H, 7.59%).

(±)-Isomenthone

(±)-Piperitone (14.0 g), b. p. 98~100°/9.5 mm, d_D^{20} 0.9418, n_D^{20} 1.4864, MR. 46.44 (Calcd. 45.72), $[\alpha]_D^{20}$ −1.6° (homog.). λ_{max} 235mμ, E_{max} 14621 (in methanol), separated from Japanese peppermint oil, was dissolved in ethanol (50 ml) and hydrogenated over 5%-palladium bariumsulfate (3.8 g) at 33° under 74 kg/cm² pressure maximum, until it was saturated. The product (13.4 g) was obtained as a colorless oil with a characteristic odor of menthone; b. p. 95~97°/19 mm, d_4^{25} 0.8962, n_D^{25} 1.4512, MR. 46.36 (Calcd. 46.19), $[\alpha]_D^{25}$+1.2° (homog.), λ_{max} 280 mμ, E_{max} 44 (in methanol). This fraction seemed to be essential (±)-isomenthone, by comparison of its infrared spectrum with that reported by Naves[8], and was used for the next step without further purification.

(±)-Isomenthol and its Derivatives

(±)-Isomenthone (9.9 g) was reduced with sodium in aqueous ammonia according to the above-mentioned procedure. The reaction temperature was maintained at 0~3°. The reduction product (8.8 g, Yield 87.7%), namely menthol mixture, was obtained by the usual way as a colorless semi-solid; b. p. 72~75°/4 mm.

(i) The menthol mixture (5.49 g) gave crude 3,5-dinitrobenzoate (12.00 g, Yield 97.5%), m. p. 109~117°. Three crystallizations of this ester from methanol gave a homogeneous fraction (6.04 g, Yield 50.3% from crude ester), m. p. 121°, unchanged by recrystallizations from *n*-hexane. Although this melting point (121°) agreed well with that of (±)-menthyl 3,5-dinitrobenzoate reported by Read[1], the infrared spectrum of the regenerated mentohl, m. p. 37.7~38.0°, showed the presence of chief peaks characteristic of menthol, but absorption bands at 829, 855, 924, 946 and no band at 1096 cm^{-1} indicated the presence of isomenthol. Therefore, this 3,5-dinitrobenzoate was decided to be a mixed crystal of (±)-menthyl and (±)-isomenthyl 3,5-dinitrobenzoate. An attempt made to separate (±)-isomenthyl 3,5-dinitrobenzoate by this method was unsuccessful.

(ii) Then, pure (±)-isomenthol was obtained by the recrystallization of menthol mixture from nitromethane. After 3 recrystallizations, the menthol mixture (2.76 g) gave pure (±)-isomenthol (0.97 g, Yield 35.2%), m. p. 52~53°, *Anal.* Found: C, 76.95; H, 12.96; Calcd. for $C_{10}H_{20}O$: C, 76.86; H, 12.90%. The infrared

7) P.B. Report, BIOS/DOCS/2905/2429/2

8) Y.R. Naves et J. Lecomte, *Bull. soc. chim. France*, **1955**, 792.

9) H. Ueda and T. Mitsui, *J. Agr. Chem. Soc. Japan*, **28**, 945 (1954).

spectrum was completely similar to that of (+)-isomenthol[9] in carbon disulfide solution.

(±)-Isomenthol gave 3,5-dinitrobenzoate, m.p. 129~130°, mixed m.p. 107~118° with (±)-neomenthyl 3,5-dinitrobenzoate, (Found: C, 58.45; H, 6.51; Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%); and *p*-nitrobenzoate, m.p. 58~59°, (Found; C, 66.97; H, 7.82; Calcd. for $C_{17}H_{23}O_4N$: C, 66.86; H, 7.59%).

(±)-Neomenthol Derivatives

(±)-Neomenthol was prepared from (±)-menthone according to the procedure of Read[5].

(±)-Neomenthyl 3,5-dinitrobenzoate, m.p. 130~131°, (Found: C, 58.45; H, 6.34; Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%). (±)-Neomenthyl *p*-nitrobenzoate, m.p. 78~79°, (Found: C, 67.05; H, 7.76; Calcd. for $C_{17}H_{23}O_4N$: C, 66.86; H, 7.59%).

(±)-Neoisomenthyl 3,5-Dinitrobenzoate

(±)-Neoisomenthyl 3,5-dinitrobenzoate, m.p. 72.5~73°, was prepared from a mixture of (+)-neoisomenthyl 3,5-dinitrobenzoate, m.p. 99.5~100.5°[9], and (−)-neoisomenthyl 3,5-dinitobenzoate, m.p. 99.5~100°*.

Acknowledgements The author is indebted to Prof. S. Takei, Prof. T. Mitsui, Prof. M. Nakajima and Assoc. Prof. T. Hashizume, University of Kyoto, for their kind advice and suggestions. He also wishes to express his sincerest thanks to Prof. S. Shimizu, Shinshu University, and Dr. Y. Inouye, Institute for Chemical Research, University of Kyoto, for their kind guidance throughout this series, and to Mr. K. Fujimoto and Miss. Y. Fukumoto for their assistance in carrying out this work.

* (−)-Neoisomenthyl 3,5-dinitrobenzoate was prepared from (−)-isomenthone by reduction with lithium aluminum hydride.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 508~510, 1960]

Studies on the Reduction of Terpenes with Sodium in Aqueous Ammonia

Part VII. On the Reduction of (+)-Menthone Enol Acetate*

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Received December 29, 1959

(+)-Menthone enol acetate was prepared from (−)-menthone. It was found that this compound can be reduced to menthols, 79% of (−)-menthol and 21% of (+)-isomenthol, through menthylacetate, or enolic menthone (menthone), by the treatment with sodium in aqueous ammonia, i.e., a novel method of reduction employed in this series.

(+)-Menthone enol acetate (I) has been prepared from both (−)-menthone and (+)-isomenthone by several groups of workers[1~3]. However, this compound can not be catalytically reduced under high pressure in the presence of Pt, Cu, Ni or Raney-Ni catalysts. The difficulties encountered in reducing this compound should be ascribed to steric hindrance.

In the present investigation, (+)-menthone enol acetate was obtained from (−)-menthone

* For Part VI see This Bulletin, **24**, 506 (1960).

1) C. Mannich und V.H. Haencu, *Ber.*, **570**, 564 (1908).

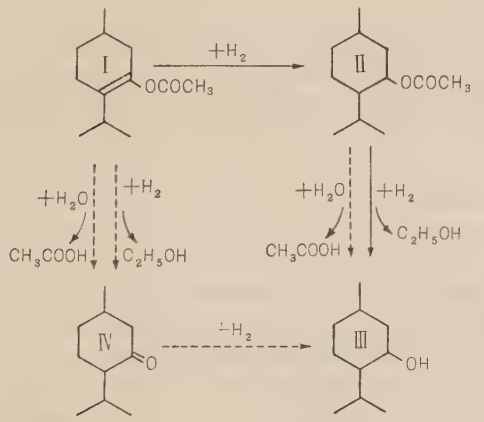
2) H. Schmidt, *Ber. Schimmel*, 124 (1938); *C.A.*, **33**, 3331 (1939).

3) F. Nerdel und W. Doll, *Ber. Shimmel*, 116 (1939); *Chem. Ztg.*, **I**, 218 (1940).

by acetylation with acetic anhydride in the presence of sulphuric acid as catalyst. It was found that this compound can be quantitatively determined by the bromine method, using pyridine-bromine-sulphate.

It was of stereochemical interest to find why this compound absorbs bromine, whereas it resists catalytic hydrogenation. In order to explain this fact, it seemed reasonable to assume that the double bond of menthone enol acetate easily accepts *trans*-addition, but resists *cis*-addition.

From the reason that the reduction with sodium in aqueous ammonia employed in this series differs mechanistically from the catalytic hydrogenation, (+)-menthone enol acetate was treated with sodium in aqueous ammonia. The resulting product was shown to consist of 88.4% of menthol (III), 5.6% of menthylacetate (II), 3.7% of menthone (IV) and 2.3% of unreacted



menthone enol acetate (I). The optical rotations of this product and its oxidation product revealed that this menthol fraction is composed of 79% of (−)-menthol and 21% of (+)-isomenthol. Acetic acid (18.4% based on enol acetate) and ethyl alcohol were also detected in the reduction mixture.

This experimental result showed that the majority of menthone enol acetate is reduced to menthol through menthylacetate or enolic menthone (menthone) by the treatment with sodium in aqueous ammonia, though a minor amount is hydrolysed.

Since neomenthol can be inverted to menthol quantitatively through menthone as described previously[4], the result of this investigation suggests the possibility of commercial inversion of all menthol isomers to menthol.

EXPERIMENTAL

All melting and boiling points were uncorrected. Iodine number was determined by Rosenmund-Kuhnhenn's method, using pyridine-bromine-sulphate[5]. Menthone was determined by the hydroxylamine method as described in the previous paper[4].

(+)-Menthone Enol Acetate

A mixture of (−)-menthone (51 g), $[\alpha]_D^{22}$ −28.3°, acetic anhydride (100 ml) and concentrated sulphuric acid (10 ml) was heated under reflux for three hours. The acetylation product (47.1 g) was obtained as a colorless oil with a characteristic odor of menthylacetate; b. p. 71~82°/4 mm, d_4^{17} 0.9451, n_D^{17} 1.4659, $[\alpha]_D^{17}$+41.21° (homog.), EV. 183.54. Fractional distillation of the product (87.6 g) in Widmer Column (length 30 cm) gave the following fractions; (i) b. p. 66~71.3°/3 mm, 27.0 g, $[\alpha]_D^{16}$+19.05°, EV. 46.03, menthone; (ii) b. p. 71.3~76.0°/3 mm, 16.1 g, $[\alpha]_D^{16}$+48.61°, EV. 156.02, menthone and menthone enol acetate; (iii) b. p. 76.0~77.0°/3 mm, 31.7 g, $[\alpha]_D^{16}$+69.86°, EV. 240.63, menthone enol acetate; (iv) b. p. 88~97°/3 mm, 10.6 g, $[\alpha]_D^{16}$+20.61°, EV. 280.76, menthone enol acetate and diacetate (?); (v) residue, 0.3 g; the distillation loss being 1.9 g.

Fractions (ii) and (iii) (44.2 g) were treated with semicarbazide-hydrochloride according to the procedure reported[6]. The contained menthone was removed as semicarbazone (6.3 g), and the fraction which remained unchanged was distilled to give pure (+)-menthone enol acetate (35.6 g); b. p. 78~79°/3.5 mm, d_4^{20} 0.9407, n_D^{20} 1.4562, $[\alpha]_D^{20}$+71.05° (homog.), MR. 56.73 (Calcd. 56.60), EV. 280.54 (Calcd. 285.84), iodine number 126.73 (Calcd. 129.33). *Anal.* Found: C, 73.32; H, 10.25; Calcd. for $C_{12}H_{20}O_2$: C, 73.43; H, 10.27%.

Hydrolysis of (+)-menthone enol acetate (5.75 g) with N/2-alcoholic potassium hydroxide for one hour gave a menthone mixture (3.51 g); b. p. 54~55°/4 mm, d_4^{22}

4) H. Ueda, Y. Shibahara and S. Shimizu, This Bulletin, **23**, 376 (1959).
5) H. Ueda and S. Shimizu, This Bulletin, **23**, 524 (1959).
6) H. Ueda and S. Shimizu, This Bulletin, **23**, 380 (1959).

0.8941, n_D^{22} 1.4515, MR. 46.49 (Calcd. 46.19), $[\alpha]_D^{22}$ +11.6° (homog.). Optical rotation showed that it consists of 67.6% of (−)-menthone and 32.4% of (+)-isomenthone.

Reduction of (+)-Menthone Enol Acetate

(+)-Menthone enol acetate (9.63 g) was reduced with sodium (7.5 g) in aqueous ammonia according to the procedure described previously[4]. The reaction temperature was maintained at 0~3°. The product (7.23 g) was obtained in usual way; b. p. 78~80°/5 mm, m. p. 31.7~33.1°, $[\alpha]_D^{21}$ −31.01° (c, 38.08, in ethanol), menthone content 3.72%, iodine number 2.99, EV. 22.38. These values showed that this reduction product consists of menthol (88.38%), menthylacetate (5.58%), menthone (3.72%) and unreacted menthone enol acetate (2.32%).

Hydrolysis of this reduction product (10.2 g) gave a menthol-menthone mixture (9.3 g); b. p. 81.7~82.0°/7 mm, m. p. 32.1~35.7°, $[\alpha]_D^{19}$ −31.40° (c, 37.62, in ethanol), menthone content 5.41%.

The menthone content corresponded well with the theoretical value derived from menthone and menthone enol acetate in the original reduction product. The menthol-menthone mixture (14.2 g) was treated with semicarbazide-hydrochloride by the same method as described above. The contaminated menthone was removed as semicarbazone (0.6 g), and the fraction which remained unchanged in the above treatment was distilled to give a menthol mixture (12.7 g).

Menthol Mixture

The menthol mixture obtained as described above had the following properties; b. p. 74.5~75.0°/4 mm, m. p. 34.8~37.7°, $[\alpha]_D^{14}$ −33.22° (c, 24.50, in ethanol). From the melting point and optical rotation, this menthol mixture seemed to be composed of 77.9% of (−)-menthol and 22.1% of (+)-isomenthol.

Oxidation of the menthol mixture (3.98 g) with Beckmann's chromic acid at 50° gave a corresponding menthone mixture (3.44 g); b. p. 63.5~64.0°/6 mm, d_4^{17} 0.8969, n_D^{17} 1.4521, MR. 46.40 (Calcd. 46.19), $[\alpha]_D^{17}$ −2.93° (homog.). Optical rotation showed that it consists of 79.4% of (−)-menthone and 20.6% of (+)-isomenthone. This ratio agreed well with that of the menthol isomers described above.

The menthol mixture (1.00 g) was converted into 3,5-dinitrobenzoate (2.22 g). Four crystallizations from *n*-hexane gave pure (−)-menthyl 3,5-dinitrobenzoate (1.33 g, Yield 59.0%), m. p. 152~153°, undepressed by admixture with an authentic sample.

Identification of Ethylalcohol and Acetic Acid

(+)-Menthone enol acetate (10.0 g) was reduced by the same method as described above. The resulting mixture was extracted with ether continuously for six hours.

The extract was fractionally distilled to give fraction (22.2 g), b. p. 45~72° (bath temp. 145°). This fraction (12.7 g) was esterified with 3,5-dinitrobenzoylchloride in pyridine in the usual manner. Crystallization of the crude ester from *n*-hexane gave ethyl 3,5-dinitrobenzoate (245 mg) as pale yellow needles, m. p. 92°, unchanged on admixture with an authentic sample.

After removal of ammonia by steam distillation, the ether insoluble part was acidified by adding 20% of sulphuric acid (100 ml) dropwise, and was steam distilled until 100 ml of the distillate required not more than 0.05 ml of N/2-alcoholic potassium hydroxide. Total distillate (659 ml) contained of 541 mg of acetic acid. This value showed that 18.4% of (+)-menthone enol acetate (or menthylacetate) is hydrolysed in this reduction process.

Acknowlegements The author is indebted to Prof. S. Takei, Prof. T. Mitsui, Prof. M. Nakajima and Assoc. Prof. T. Hashizume, University of Kyoto, for their kind advice and suggestions. He also wishes to express his sincerest thanks to Prof. S. Shimizu, Shinshu University, and Dr. Y. Inouye, Institute for Chemical Research, University of Kyoto, for their kind guidance throughout this series, and to Mr. K. Fujimoto, Miss M. Kashimoto and Miss. Y. Fukumoto for their assistance in carrying out this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 511~516, 1960]

Spectrophotometric Determination of the Indicator Time Test Value of Beer*

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Received January 16, 1960

Determination of the I.T.T. (Indicator Time Test) value of beer has so far been conducted by the visual measurement. However, this visual judgement of the critical point, at which 80% of added dyestuff is accurately decolorized, is very difficult. We attempted to estimate the I.T.T. value more correctly using a spectrophotometer. On the basis of the following facts and observations, spectrophotometric determination of the I.T.T. value has been proposed. The absorption maximum of 2,6-dichlorophenolindophenol was observed at 520 mμ, but the absorption of beer was hardly ever recognized at this wave length. Walpole's buffer solution was used for stabilizing the dyestuff at a pH similar to that of beer. Ethanol contained in beer did not affect the absorption of dyestuff. It was also found that the optical density of the dyestuff was proportional to the concentration both in water and in beer.

A number of papers for determining the oxidation-reduction potential of beer have been published. Since the oxidation-reduction system of beer is composed of unknown oxidants and reductants, and accordingly irreversible and highly unpoised, the true oxidation-reduction potential of beer can not be theoretically estimated. Therefore, the determination of the oxidation-reduction potential or of the rH value, which has been estimated colorimetrically[1~4] or electrometrically[5~7], is based on a tacit assumption. In fact, the expression of rH is erroneous and its use is at present entirely opposed[8,9]. However, the rH value is now employed conveniently in brewing, from the standpoint that it does not really represent the reducing potential but it indicates an apparent reducing level for the process control[7,10~14]. As mentioned above, there has been no alternative but to use the I. T. T. value as a reasonable expression of the state of oxidation of beer. The determination of the I. T. T. value is extremely simple for 2,6-dichlorophenolindophenol is inactive to dissolved oxygen in beer, as this oxidation-reduction indicator has a higher potential than that of beer.

However, the I. T. T. value is taken in seconds by a visual measurement, comparing the fading color of the dyestuff added in the sample with that of a standard. This visual judgement involves considerable individual error perhaps due to fatigue of visual power.

In order to overcome this defect, the spectrophotometric determination of the I. T. T. value was attempted and a new method was established.

* The content of this report was previously presented in the "*Kirin Kiyô*". September (1954), a bulletin of our Research Laboratories, not open to the public.

1) J. de Clerck, *Bull. Assoc. Etud. Ecole Super. Brasserie*, **34**, 55, 78 (1934).
2) B. Hartong, *Wochschr. Brau.*, **51**, 409 (1934).
3) S. Laufer, *Am. Brewer*, **69**, 15 (1936).
4) I. Preece, *J. Inst. Brewing*, **42**, 27 (1936).
5) F. Mendlik, *Wochschr. Brau.*, **51**, 305 (1934).
6) F. Mendlik, *Wochschr. Brau*, **52**, 417 (1935).
7) H.D. Schulz, *Brauwiss.*, **11**, 193 (1953).
8) P.P. Grand and I. Stone, *J. Inst. Brewing*, **45**, 253 (1939).
9) H. Heyer and E. Paukner, *Brauwiss.*, **12**, 208 (1952).
10) V. Schreiber and H. Moder, *Abst. J. Inst. Brewing*, **57**, 209 (1951).
11) A. Szilivinyi, *Brauwiss.*, **1**, 8 (1951).
12) F. Knorr, *Brauwiss.*, **2**, 17 (1953).
13) K. Raible, *Brauwiss.*, **6**, 122 (1955).
14) F. Weinfurtner, A. Uhl and R. Pöhlmann, *Brauwiss.*, **8**, 166, 192 (1955).

SPECTROPHOTOMETRY FOR DETERMINATING THE I. T. T. VALUE

All the procedures including the sampling are carried out at 25°C.

The optical density is measured at 520 mμ by Beckman spectrophotometer model DU using the 10 mm absorption cells at 25°C. For this purpose, the cell compartment is maintained at 25°C, using a thermospacer.

1) Optical Density of 80% Decoloration Standard.

Fifty percent decoloration solution is prepared by adding 0.125 ml of 0.005 M 2,6-dichlorophenol-indophenol into 10 ml of Walpole's buffer solution having a similar pH to beer. When the optical density is multiplied by two-fifths, that of 80% decoloration standard is obtained.

2) Decoloration Curve of Beer.

A 0.25 ml portion of 0.005 M 2,6-dichlorophenol-indophenol is added to 10 ml of beer. When the optical density of this mixture is measured from time to time after being transferred to the absorption cell, the decoloration curve is gained.

3) Blank Beer Line.

To several test tubes each containing 10 ml of beer is added 0.25 ml of 0.005 M 2,6-dichlorophenol-indophenol solution and immerged in a thermostat at 25°C. At definite intervals, 0.05 ml of 2% ascorbic acid in Walpole's buffer solution (pH 4.4) is added to each tube and the optical density is measured. When beer is decolorized with ascorbic acid immediately after the addition of 2,6-dichlorophenol-indophenol, the optical density of the decolorized beer is similar to that of beer itself. However, the longer the dyestuff is kept in contact with beer, to the greater extent increases the optical density of the decolorized beer, owing to turbidity appeared.

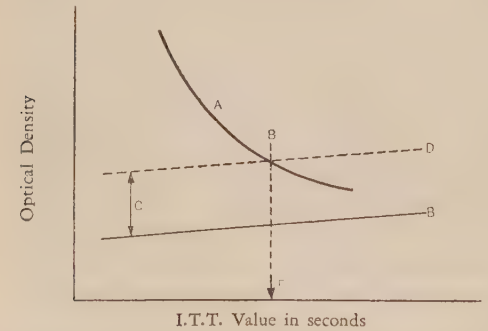

FIG. 1. Deduction of I.T.T. Value by Drawing

Curve A : Decoloration curve of beer.
Line B : Blank beer line.
Distance C : Optical density of 80% decoloration standard.
Line D : Blank beer line plus optical density of 80% decoloration standard.
Point F : I.T.T. value deduced.

4) Deduction of the I. T. T. Value.

When line D is drawn in Fig. 1 which indicates the sum of the optical density of 80% decoloration standard, C and of blank beer line, B, it intersects the decoloration curve A at point E. The I. T. T. value is obtained from the intersection F where a perpendicular line drawn through point E intersects the abscissa.

EXPERIMENTAL

1) Absorption Curves of Beer and of Dyestuff Solution.

As shown in Fig. 2, 2,6-dichlorophenol-indophenol solution has its absorption maximum at 520 mμ, but at this wave length beer had a hardly recognizable optical density of about 0.05. The dyestuff solution decolorized with ascorbic acid did not show the absorption at a longer wave length than 400 mμ.

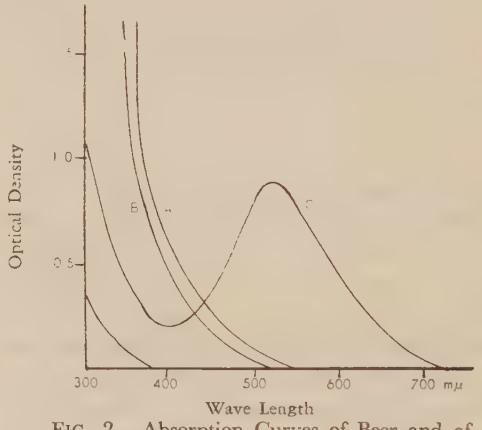

FIG. 2. Absorption Curves of Beer and of 2,6-Dichlorophenol-Indophenol

A, B : Beer
C : Dyestuff solution
D : 10 ml of Walpole's buffer solution (pH 4.4) plus 0.25ml of 0.0045 M dyestuff solution plus a sufficient amount of ascorbic acid.

In order to investigate the effect of pH on the absorption of the dyestuff solution, 0.25 ml of 0.005 M 2,6-dichlorophenol-indophenol solution was added to 10 ml of McIlvaine's buffer solutions adjusted to pH 4.0, 4.5, 5.0, 5.5 and 6.0.

The absorption curves at different pH's are shown in Fig. 3. Their maxima are almost identical at 520 mμ in the range of pH 4 to 5, but they shift toward longer wave length, when pH of the solution increases.

From the result, it was desirable that the best condition for determining the I. T. T. value of beer was to measure the optical density at 520 mμ in the range of pH 4 to 5.

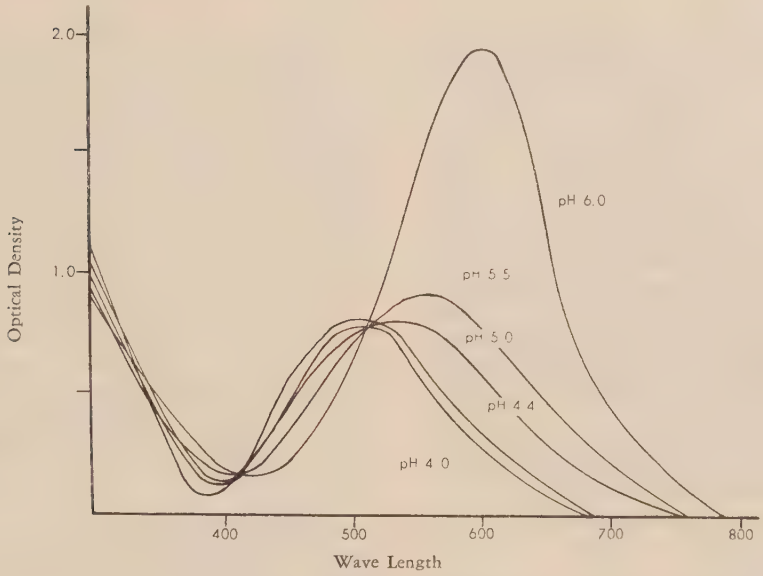

FIG. 3. Absorption Curves of 2,6-Dichlorophenol-Indophenol in Buffer Solutions Ranging from pH 4 to 6

2) Selection of Buffer Solution for Stabilizing the Dyestuff Solution.

For stabilizing 2,6-dichlorophenol-indophenol solution, the degradation of dyestuff in various buffer solutions at pH's 4.4, and 7.0 was investigated.

As summarized in Table I, the dyestuff solution is stable in a neutral solution, but is slightly unstable in an acid medium. Degree of degradation of dyestuff at pH 4.4 gives the different results, as expected, in response to the composition of buffer solution. The degradation is smaller in Walpole's than in McIlvaine's buffer solution. Also, the rate of degradation is independent of the concentration of the dyestuff solution. Thus, when Walpole's buffer solution was employed, the degradation was scarecely observed within the time consumed for determing the I. T. T. value.

TABLE I. EFFECT OF COMPOSITION OF BUFFER SOLUTION ON STABILITY OF 2,6-DICHLOROPHENOL-INDOPHENOL

A definite amount of 0.005 M dyestuff solution was added to 10 ml of buffer solution. Degree of degradation at 22°C after 60 min. was calculated.

pH	Buffer solution	0.005 M Dyestuff		
		0.250 ml	0.125 ml	0.050 ml
4.4	McIlvaine's	5.6%	5.5%	6.0%
	Walpole's	—	2.5	2.9
7.0	Sørensen's	—	0.0	—
	Distilled water	—	0.0	—

3) Effect of Ethanol on the Light Absorption of Dyestuff Solution.

Ethanol has no absorption in the range of wave length used in this experiment but it may affect the absorption of 2,6-dichlorophenol-indophenol solution.

In order to see this effect, ethanol was added to Walpole's buffer solution (pH 4.4) to produce 5 or 10% by volume. The optical densities of these buffer solutions at 520 mμ were compared with those of the buffer solutions containing no ethanol.

It was observed that the buffer solution containing 5 or 10% ethanol showed only 2 or 3% increase in the optical density. So the effect of ethanol in beer on the determination of I. T. T. value would be negligible.

4) Relation between the Concentration of Dyestuff and the Optical Density.

The relation between the concentration of 2,6-dichlorophenol-indophenol and its optical density was investigated by the following two experiments. One was conducted by adding a definite amount of the dyestuff solution to Walpole's buffer solution, whereas the other by decoloration of the dyestuff solution by the addition of a definite amount of ascorbic acid. As shown in Figs. 4 and 5, a straight-line correlation was gained in both

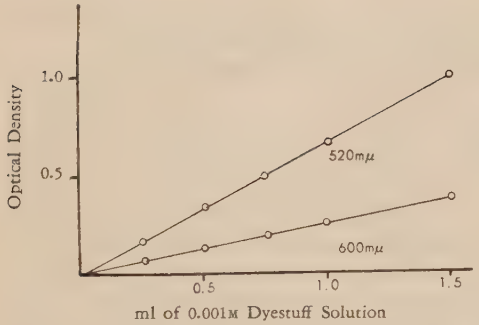

FIG. 4. Relationship between the Concentration of 2,6-Dichlorophenol-Indophenol and its Optical Density

Various amount of 0.001 M dyestuff were added to 10 ml of Walpole's buffer solution and the optical density was measured.

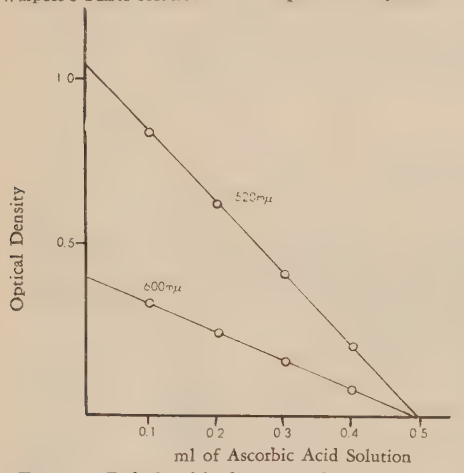

FIG. 5. Relationship between the Concentration of 2,6-Dichlorophenol-Indophenol and its Optical Density on the Decoloration of the Dyestuff

Concentration of ascorbic acid was adjusted, so as to decolorize perfectly 0.25 ml of 0.005 M dyestuff with 0.5 ml of ascorbic acid solution. Various amounts of ascorbic acid were added to a mixture of 10 ml of Walpole's buffer solution and 0.25 ml of 0.005 M dyestuff. The optical density of partly decolorized dyestuff solution was estimated.

runs.

Then, in order to examine whether Beer's law holds when beer and dyestuff are mixed, the following experiments were conducted. Because, beer, having a considerably strong reducing power, may decolorize 2,6-dichlorophenol-indophenol instantly to make the spectrophotometric estimation impossible.

In order to diminish the reducing power of beer with an oxidizing agent, 150 ml of old beer stored for over one year was degassed, treated with 3.75 ml of 0.005 M

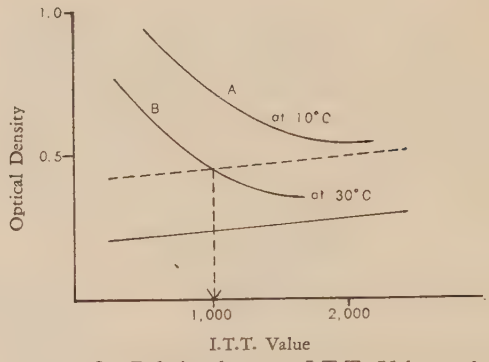

FIG. 6. Relation between I.T.T. Value and Temperature

Rate of decoloration of the dyestuff depends on temperature. Line C intersects curve B at 1,000 sec., but not curve A, within 2,000 sec.

2,6-dichlorophenol-indophenol, allowed to stand overnight at room temperature to decrease its reducing power and filtered through a Büchner funnel with a thin layer of diatomaceous earth.

When the optical density was measured immediately after 10 ml of the beer thus treated was mixed with various amounts of dyestuff, a straight-line correlation was obtained. When fresh beer, instead of old beer, was treated similarly with or without the removal of turbidity through filtration, the same results were obtained.

Therefore, it was concluded that the optical density of dyestuff added to beer was in accord with Beer's law.

5) Examination of Each Procedure for Determining the I. T. T. Value.

i) Effect of temperature on the I. T. T. value.

Comparing the decoloration curves of beer at 10°C and 30°C in Fig. 6, the decoloration rate or the rate of oxidation of 2,6-dichlorophenol-indophenol was shown to be greatly influenced by temperature. For instance, the I. T. T. value at 30°C obtained from Fig. 6 is about 1000, and that at 10°C is greater than that at 30°C.

The I. T. T. value should, therefore, be measured at a definite temperature, and 25°C was chosen as the appropriate temperature according to Gray and Stone's work[8].

ii) Effect of air on the I. T. T. value.

The reason for using 2,6-dichlorophenol-indophenol was that it is inactive to dissolved oxygen, because it has a higher oxidation-reduction potential.

In order to investigate the effect of air on the I. T. T. value, the data obtained in the absence and in the

presence of air, were compared. One experiment was conducted without exposing beer to air by the use of a special equipment attached to the bottle. Beer was taken into several Tunberg tubes filled with nitrogen gas, mixed with 2,6-dichlorophenol-indophenol, which was prepacked in the side chambers of the tubes, and kept in a thermostat at 25°C. Aliquots of the mixture in the tubes were transferred to the absorption cells from time to time and the optical densities were measured as rapidly as possible. The decoloration curve in the absence of air was thus obtained.

The other experiment was carried out in contact with air. Beer was pipetted in the test tubes from the bottle, after taking off the equipment to eliminate air, and the decoloration curve was obtained. The I. T. T. values obtained from the two curves, are the same as seen in Table II.

TABLE II. EFFECT OF AIR ON ESTIMATION OF THE I.T.T. VALUE (I)

Beer was sampled from a bottle in the presence and the absence of air.

Sampling	I.T.T. Value
Air present	1160
Air absent	1150

Then, the I. T. T. values were measured when fresh beer was taken from the bottle immediately after opening its crown, and when beer remained in the bottle was poured into a beaker and allowed to stand for sixty minutes. Comparing the two values, it was shown that the values were identical as indicated in Table III. Consequently, atmospheric oxygen had no immediate effect on the estimation of the I. T. T. value, as reported by Gray and Stone[5].

TABLE III. EFFECT OF AIR ON ESTIMATION OF THE I.T.T. VALUE (II)

I.T.T. values were estimated as to the samples immediately after opening of a bottle and after standing for 60 min. in a beaker.

Sample	Immediately after opening	After standing
1	2030	2060
2	2160	2100

iii) Preparation of dyestuff solution.

The power of 2,6-dichlorophenol-indophenol decreases gradually with time even when the stock solution is kept in the cold, and eventually this dyestuff solution deteriorates so that it can not be perfectly decolorized with ascorbic acid. In such a case, the stock solution should be prepared anew. Also, it must be noticed, referring Heyer and Paukner's report[9] that the I. T. T. value is largely influenced by the purity of 2,6-dichlorophenol-indophenol.

6) Comparison between the I. T. T. Values Estimated by Spectrophotometry and by Visual Measurement.

The I. T. T. values of unpasteurized and pasteurized beer were determined at about eight and twenty-five hours after bottling, both spectrophotometrically and visually, but they are nearly identical as shown in Table IV. Then, the effect of the lapse of time after bottling on the I. T. T. value was investigated. In general, the I. T. T. values gradually increase as the time passes; for example, the I. T. T. value of unpasteurized beer was 1340 just after the bottling but increased to 1490 in fifty-eight hours after bottling. This tendency appears to be rather more marked for pasteurized than for unpasteurized beer.

TABLE IV. COMPARISON OF I.T.T. VALUES OBTAINED BY SPECTROPHOTOMETRY AND BY VISUAL MEASUREMENT

Beer	Measurement of I.T.T. value	At about 8 hrs. after bottling	At about 25 hrs. after bottling
Unpasteurized	A	1920	1910
	B	1860	2040
Pasteurized	A	1920	2010
	B	1890	2030

A: Spectrophotometry, B: Visual Measurement

CONCLUSION

A method for the spectrophotometric determination of the I. T. T. values has been established as described in the section "Spectrophotometry for Determining the I. T. T. Value" under the following conditions.

1) Maximum absorption of 2,6-dichlorophenol-indophenol was at 520 mμ at pH 4 to 5.

2) Walpole's buffer solution was the best among various buffer solutions from the standpoint of stability of 80% decoloration standard.

3) Ethanol in beer showed hardly recognizable effect on the optical density of the dyestuff.

4) The relationship between the concentration of the dissolved dyestuff and the optical density was in accord with Beer's law.

5) The decoloration of dyestuff was dependent on the temperature, and 25°C was chosen for the measurement, referring the work of Gray and Stone.

6) Atomospheric oxygen did not influence the I. T. T. value.

7) From the data above mentioned "Optical Density of 80% Decoloration Standard", "Decoloration curve of beer", and "Blank beer line" were gained. The I. T. T. value can be obtained from these diagrams.

Acknowledgement The authors would like to thank Messrs. M. Tokikuni and T. Kumazawa, the managing directors of Kirin Brewery Co. Ltd., who gave us the opportunity to publish this report. We are grateful for many suggestions and the continued interest of Dr. Y. Umeda, the President of the Research Laboratories of Kirin Brewery Co. Ltd., and Dr. Y. Okuda, a member of the Japan Academy and the former president of our Research Laboratories.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 516~520, 1960]

Studies on Transfer of Antiseptics to Microbes and their Toxic Effect

Part IV. Adsorption of Esters of Acid Antiseptics on Yeast Cell and their Toxic Effect

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Received January 20, 1960

The transfer of alkyl esters of sorbic, benzoic and salicylic acid from the medium to bakers' yeast cell was investigated, and both the quantity of the ester dissolved in the lipid phase of the cell and the quantity adsorbed on the solid phase were determined.

The dissolved quantity of these esters was very great in comparison with the adsorbed quantity. At the ester concentration which gives a definite inhibiting effect on the yeast growth, the adsorbed quantity remained constant, being independent of the kind of ester, while the dissolved quantity greatly varied according to the kind of ester. From this fact it was concluded that the toxic effect of these esters, as well as esters of *p*-hydroxybenzoic acid (cf. Part III), is exclusively limited by the adsorbed quantity.

It has been assumed that the partition coefficient of lipophyl substances such as esters, amides, ethers, alcohols, phenols etc. between lipid and water is related to the biological effect[1~3]. However, in the previous report[4], it was considered that the toxic effect of esters of *p*-hydroxybenzoic acid on the yeast growth is dependent upon the quantity of the ester adsorbed on the solid phase of the cell and is independent of the quantity dissolved in the lipid phase.

In the present investigation, the partition and the adsorption of alkyl esters of sorbic, benzoic and salicylic acid were examined in relation to their toxic effect, and it was confirmed that the toxic effect of these esters, as well as esters

1) K.H. Meyer, *Trans. Farady Soc.*, **32**, 1062 (1937).
2) J.F. Danielli, "Cell Physiology and Pharmacology" Elsevier (New York) 1950, p. 97.
3) K. Hirai, *J. Pharm. Soc. Japan*, **77**, 1279 (1957).
4) S. Oka, This Bulletin, **24**, 412 (1960).

of *p*-hydroxybenzoic acid, is exclusively limited by the adsorbed quantity, though the partition coefficient of above-mentioned esters are far greater than those of ester of *p*-hydroxybenzoic acid[4].

EXPERIMENTAL

Experimental methods and materials were the same as those described in the previous reports[4~6].

The esters used were methyl, ethyl and *n*-butyl esters of sorbic, benzoic and salicylic acid. The concentration of these esters was spectrophotometrically determined in the aqueous solution. The wave-length and the molar optical extinction at the maximum absorption was found to practically coincide with those of the corresponding original acid in 0.2% aqueous hydrochloric acid solution[5].

RESULTS

Toxic effect of esters The minimum concentration of alkyl esters of acid antiseptics required to inhibit the yeast growth for two days was determined. As seen in Table I, the toxic effect of these esters is practically independent of the pH value of the medium. On the other hand, the toxic effect increases at the rate of about $(2.4)^N$ along with the increase of carbon number, N, in the alkyl radical of the ester as given in Table II.

Transfer of ester from medium to yeast cell The transfer of these esters from the buffer solution to the yeast cell was examined. The transfer equilibrium of the ester can be obtained within five minutes as shown in Table III, and the transfer is independent of the pH value of the medium as shown in Table IV.

Table I. Influence of pH Value on Toxic Effect of Esters

	Minimum inhibiting concentration (m-mol./kg-water)			
pH of medium	3.5	4.5	5.5	6.5
n-Butyl sorbate	0.3	0.4	0.4	0.4
Ethyl benzoate	2	3	3	3
Methyl salicylate	3	4	4	4

As the culture medium, koji extract (Ballg. 7°) which was adjusted to the desired pH with hydrochloric acid or sodium hydroxide was employed. The medium containing an ester was inoculated with washed bakers' yeast cell in 0.0003% and incubated for two days at 30°C.

5) S. Oka, This Bulletin, **24**, 59 (1960).
6) S. Oka, *ibid.*, **24**, 338 (1960).

Table II. Toxic Effect of Esters of Acid Antiseptics

	Minimum inhibiting concentration (m-mol./kg-water)			Increase rate of toxic effect by carbon number, N, in alkyl radical
	Methyl ester	Ethyl ester	*n*-Butyl ester	
Sorbic acid	5	2	0.4	$(2.33)^N$
Benzoic acid	6	3	0.4	$(2.41)^N$
Salicylic acid	4	2	0.3	$(2.38)^N$

The experimental condition was the same as in Table I. The pH value of the medium was 5.6.

Table III. Change of Transfer Ratio with Suspending Time

	Transfer ratio (kg-water/kg-cell)			
Suspending time (min.)	5	30	60	180
Ethyl sorbate	13.1	13.5	12.9	13.4
Ethyl benzoate	14.5	14.4	13.5	14.2
Ethyl salicylate	38.6	38.8	38.1	38.5

Five per cent of washed bakers' yeast cells were suspended in the buffer solution (pH 5.6) containing an ester in 1.20 m-mol./kg-water at 30°C. After a definite interval of time, a part of the suspension was taken out and the transfer ratio was determined.

Table IV. Influence of pH Value on Transfer Ratio

	Transfer ratio(kg-water/kg-cell)			
pH of medium	3.5	4.5	5.5	6.5
n-Butyl sorbate	122	125	121	123
Ethyl benzoate	14.5	14.5	14.4	14.1
Methyl salicylate	11.6	11.4	11.7	11.5

Initial concentrations of the sorbate, benzoate and salicylate were 0.2, 1.2 and 1.2 m-mol./kg-water, respectively, and the proportions of the cell to the medium were 0.01, 0.05 and 0.10 kg/kg-water.

The plot of the logarithm of the transferred quantity versus the logarithm of the equilibrium concentration gives a straight line as shown in Fig. 1. This relation is expressed by equation (1):

$$a = k(C_m)^n \qquad (1)$$

where a is the transferred quantity (m-mol./kg-cell), C_m the equilibrium concentration in the medium (m-mol./kg-water), and k and n the constants of each ester. The values of k and n are given in Table V. It is noteworthy that the value of n increases as the alkyl radical becomes higher.

The quantity transferred at the minimum inhibiting concentration was evaluated by substituting the minimum inhibiting concentration

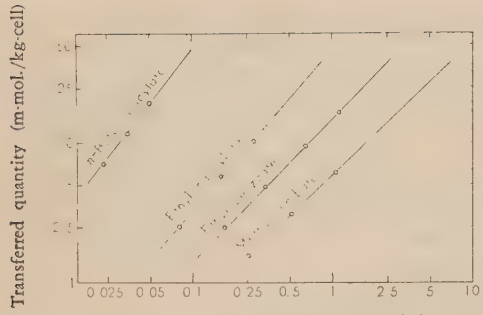

FIG. 1. Relation between Transferred Quantity and Equilibrium Concentration of Esters of Acid Antiseptics

TABLE V. RELATION BETWEEN TRANSFERRED QUANTITY AND EQUILIBRIUM CONCENTRATION

$k(n)$ (30°C)

	Methyl ester	Ethyl ester	*n*-Butyl ester
Sorbic acid	5.8 (0.95)	13.5 (0.99)	135 (1.02)
Benzoic acid	5.8 (0.98)	14.5 (1.02)	155 (1.08)
Salicylic acid	12.0 (1.08)	46.0 (1.15)	850 (1.27)

The relation between the transferred quantity, a (m-mol./kg-cell), and the equilibrium concentration in the medium, C_m (m-mol./kg-water), can be expressed by following Freundlich's adsorption isotherm.

$$a=k(C_m)^n$$

TABLE VI. INFLUENCE OF OSMOTIC PRESSURE ON TRANSFER

	$k(n)$		Ratio of dissolved quantity in cell to equilibrium conc. in medium
	Original buffer	2 mol./kg-water glycerin added	
Methyl sorbate	5.8 (0.95)	5.4 (0.94)	0.7
Ethyl benzoate	14.5 (1.02)	14.0 (1.03)	0.8
n-Butyl salicylate	850 (1.27)	850 (1.27)	0

The ratio in the last column of this table was evaluated by dividing the difference of k value in the original buffer and that in the buffer with glycerin by the relative decrease of the water content of the cell (0.6)[6].

for C_m in equation (1) as given in Table X.

Dissolution of ester in inner cell fluid From the influence of osmotic pressure on the transfer of antiseptics, the ratio of the quantity dissolved in the inner cell fluid to the equilibrium concentration in the medium can be evaluated as described in the previous reports[4,6]. From Table VI it is seen that in the cases of these esters the evaluated ratio of the dissolved quantity does not exceed 1. Judging from the fact that these esters can not be ionized in the inner cell fluid, the concentration of these esters in the inner cell fluid is considered to be equal to that of the medium as in the case of esters of *p*-hydroxybenzoic acid[4]. Hence, the quantity dissolved in the inner cell fluid is given by equation (2):

$$a_d=0.65C_m \qquad (2)$$

where a_d is the dissolved quantity (m-mol./kg-cell), C_m the equilibrium concentration in the medium (m-mol./kg-water), and the value of 0.65[6] the water content of the cell (kg/kg-cell).

The quantity dissolved at the minimum inhibiting concentration was evaluated from equation (2) and is given in Table X.

Dissolution of ester in lipid phase of cell In order to evaluate the concentration of these esters transferred into the lipid phase of the cell, the partition of the ester between soy bean oil and water was examined. The plot of the logarithm of the concentration of the ester in the oil phase versus the logarithm of the equilibrium concentration in the water phase gives a straight line as shown in Fig. 2. This relation is expressed by equation (3):

$$p=k'(C_m)^{n'} \qquad (3)$$

where p is the concentration of the ester in the oil phase (m-mol./kg-oil), C_m the equilibrium concentration in the water phase (m-mol./kg-water), and k' and n' the constants of each ester. The partition coefficient, P, is defined

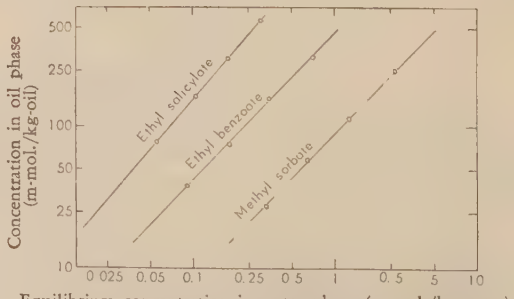

FIG. 2. Partition of Esters of Acid Antiseptics between Soy Bean Oil and Water

by equation (4):

$$P=p/C_m=k'(C_m)^{(n'-1)} \quad (4)$$

Values of k' and n' are given in Table VII. From the fact that the value of n' exceeds 1, it is seen that the partition coefficient is a function of the equilibrium concentration, and that the partition of these esters does not obey the usual partition equation. In this case, it is noteworthy that the value of n' approches the corresponding value of n of equation (1) when the value of k' becomes greater.

Supposing that the partition coefficient between the oil and water is practically equal to that between the yeast lipid and water, the partition coefficient at the minimum inhibiting concentration increases at the rate of about $(3.9)^N$ along with the increase of carbon number, N, in the alkyl radical, and under the same inhibiting condition, the concentration of the ester in the yeast lipid greatly increases as the alky radical becomes higher as seen in Table VIII.

DISCUSSION

As seen in Table V and VII, the value of k in equation (1) and also the value of k' in equation (3) are increased as the alkyl radical becomes higher, and the value of n is nearly equal to the corresponding value of n' when the value of k' becomes greater. Accordingly, it is considered that the transfer of the ester apparently depends more closely upon the partition precedure when the partition coefficient becomes greater, and that the yeast lipid plays the same role as soy bean oil as the solvent of these esters.

Supposing that at the minimum inhibiting concentration, the transferred quantity of butyl salicylate is equilibrated by the partition, the quantity of the yeast lipid as the solvent can be evaluated to be as great as 0.01697 kg/kg-cell by dividing the transferred quantity (196 m-mol./kg-cell (Table X)) by the corresponding concentration of the ester in the lipid phase (11,550 m-mol./kg-oil (Table VIII). (In this case, the quantity dissolved in the inner cell fluid is negligibly small (Table X)). Thus, the quantity of each ester dissolved in the lipid phase of the cell is evaluated by multiplying the concentration in the lipid phase (Table VIII) by 0.01697 kg/kg-cell. When the evaluated value of the quantity dissolved in the lipid phase and in the inner cell fluid are subtracted from the total quantity transferred to the cell, the residuals obtained are in the range of 10.4 ~17.4 m-mol./kg-cell as given in Table IX. These values give the approximate quantity of the esters adsorbed on the solid phase of the cell.

Assuming that butyl salicylate is also adsorbed

Table VII. Partition of Ester between Soy Bean Oil and Water

k' (n') (30°C)

	Undissociated acid	Methyl ester	Ethyl ester	*n*-Butyl ester
Sorbic acid	2.0 (2.00)	89 (1.04)	310 (1.14)	7,100 (1.22)
Benzoic acid	5.3 (1.34)	131 (1.00)	470 (1.05)	7,850 (1.11)
Salicylic acid	16.0 (1.33)	500 (1.11)	2,300 (1.17)	55,000 (1.28)

The relation between the concentration of ester in the oil, p (m-mol./kg-oil), and the equilibrium concentration in water, Cm (m-mol./kg-water), can be expressed by the following equation.

$$p=k'(C_m)^{n'}$$

Table VIII. Concentration of Esters in Lipid Phase at Minimum Inhibiting Concentration

	Concentration of ester in lipid phase (m-mol./kg-lipid)			Increase rate of partition coeff. by carbon number, N, in alkyl radical
	Methyl ester	Ethyl ester	*n*-Butyl ester	
Sorbic acid	473 (95)	685 (343)	2,310 (5,775)	$(3.93)^N$
Benzoic acid	786 (131)	1,490 (497)	2,830 (7,085)	$(3.78)^N$
Salicylic acid	2,330 (583)	5,180 (2,590)	11,550 (38,500)	$(4.04)^N$

Numbers in the brackets are the partition coefficient at the minimum inhibiting concentration in the water phase.

Table IX. Adsorbed Quantity Evaluated at Minimum Inhibiting Concentration under Assumption that 0.01695 kg/kg-cell of Yeast Lipid Relates to Partition of Ester

	Adsorbed quantity (m-mol./kg-cell)		
	Methyl ester	Ethyl ester	*n*-Butyl ester
Sorbic acid	15.5	14.1	13.5
Benzoic acid	17.4	17.2	9.1
Salicylic acid	11.7	10.4	—

to the same extent as other esters (13.6 m-mol./kg-cell as the mean value of the quantities given in Table IX), the quantity transferred in relation to the partition is 182.2 m-mol./kg-cell (Table X) and the quantity of the lipid (0.01697 kg/kg-cell) corrected is as great as 0.01577 kg/kg-cell*. By using this corrected value the corrected quantity of these esters dissolved in the yeast lipid can be evaluated as given in Table X.

Thus, it is seen that the partition coefficient increases at the rate of obout $(3.9)^N$, though the toxic effect estimated by the reciprocal of the minimum inhibiting concentration increases at the rate of about $(2.4)^N$ along with the increase of the carbon number, N, of the alkyl radical, and that the concentration of the ester dissolved in the yeast lipid varies to a great extent with the kind of alkyl radical under the same inhibiting condition (Table VIII). Accordingly, it is conclued that the toxic effect is independent of the quantity of the ester dissolved in the lipid phase as well as in the inner cell fluid (Table X).

By subtracting the quantities dissolved in the lipid phase and the inner cell fluid from the total quantity transferred, the corrected adsorbed quantity can be evaluated. As seen in the last column of Table X, the adsorbed quantities thus evalusted are practically constant (13~19 m-mol./kg-cell) under the same inhibiting condition. Accordingly, it is concluded that the adsorbed quantity is the limiting factor of the toxic effect of these esters**.

Acknowledgements The author wishes to express his sincere thanks to the late Emeritus Prof. R. Sasaki and Prof. Y. Sakurai of Tokyo University for their interest shown in this work. Thanks are also to Dr. C. Hata, President of this Institute, for his support and encouragement in this work. The author is greatly indebted to Dr. M. Fujimaki, Dr. S. Okimasu, Dr. R. Nōmi and Mr. K. Shimizu for their kind advice.

Table X. Distribution of Transferred Ester in Yeast Cell at Minimum Inhibiting Concentration

	Transferred quantity (m-mol./kg-cell)	Dissolved quantity in inner fluid (m-mol./kg-cell)	Dissolved quantity in lipid phase (m-mol./kg-cell)	Adsorbed quantity on solid phase (m-mol./kg-cell)
Methyl sorbate	26.7	3.3	7.5	15.9
Ethyl sorbate	27.0	1.3	10.8	14.9
n-Butyl sorbate	53.0	0.3	36.5	16.2
Methyl benzoate	34.6	3.9	12.4	18.3
Ethyl benzoate	44.5	2.0	23.5	19.0
n-Butyl benzoate	57.4	0.3	44.6	12.5
Methyl salicylate	53.8	2.6	36.8	14.4
Ethyl salicylate	99.4	1.3	81.6	16.5
n-Butyl salicylate	196.0	0.2	182.2	13.6

* By using the mean value of the corrected adsorbed quantity (15.7 m-mol./kg-cell (Table X)), the quantity of the yeast lipid related to partition is evaluated to be as great as 0.0156 kg/kg-cell.

** In connection with this, the adsorbed quantity of sorbic, benzoic and salicylic acid required to give the same inhibiting effect was 17~24 m-mol./kg-cell[6]. In the case of esters of *p*-hydroxybenzoic acid, the adsorbed quantity required under the same condition was evaluated to be as great as 14.8~16.5 m-mol./kg-cell[4], when the adsorbed quantity was corrected by using the value of 0.0156*kg/kg-cell for the quantity of the yeast lipid related to partition. The adsorbed quantities of the above-mentioned esters are in fairly good agreement with those values already obtained. Accordingly, one can assume a similar inhibiting mechanism for all of these antiseptics. However, in the case of dehydroacetic acid an apparently small quantity of the adsorbed quantity (3.6 m-mol./kg-cell)[6] was required to give the same inhibiting effect.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 521~524, 1960]

Studies on Transfer of Antiseptics to Microbes and their Toxic Effect

Part V. Adsorption of Phenols on Yeast Cell and their Toxic Effect

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Received January 20, 1960

The adsorption of phenols such as phenol, *o*-cresol, thymol, *o*- and *p*-phenylphenol, guaiacol, eugenol, α- and β-naphthol on bakers' yeast cell was investigated in relation to their toxic effect on the yeast growth. It was found that a definite quantity of the adsorbed phenols is required in order to give a definite inhibiting effect, being independent of the kind of phenol, and it was concluded that the toxic effect depends upon the quantity of the adsorbed phenols.

The adsorbed quantity required to inhibit the yeast growth for two days was 14~19 m-mol./kg-cell in all cases of the phenols examined, and this value is in fairly good agreement with that obtained in cases of acid antiseptics and their esters (cf. Parts II, III and IV). The inhibiting mechanism of these phenols was assumed to be the same as in cases of acid antiseptics and their esters.

In the previous reports[1~3], it was found that the toxic effect of some acid antiseptics and their esters on the yeast growth depends upon the quantity adsorbed on the solid phase of the cell and is independent of the dissolved quantity in the lipid phase or in the inner cell fluid.

In the present investigation, the transfer of certain phenols from the medium to the yeast cell was investigated in relation to their toxic effect on the yeast growth, and it was found that phenols transfer from the medium to the yeast cell in the same manner as in the case of the esters[3] and the toxic effect is also exclusively limited by the adsorbed quantity. In this case, the adsorbed quantity required to give a definite inhibiting effect was in fairly good agreement with that obtained in the cases of acid antiseptics and their ester[1~3], and the inhibiting mechanism of these phenols was assumed to be the same as in the cases mentioned above.

1) S. Oka, This Bulletin, **24**, 338 (1960).
2) S. Oka, *ibid.*, **24**, 412 (1960).
3) S. Oka, *ibid.*, **24**, 516 (1960).

EXPERIMENTAL

Experimental methods and materials were the same as those described in the previous reports[1~4].

As antiseptics were used phenol, *o*-cresol, thymol, *o*- and *p*-phenylphenol, guaiacol, eugenol, α- and β-naphthol. The concentration of these phenols was spectrophotometrically determined in the aqueous solution. The wave-length and the molar optical extinction at the maximum absorption are illustrated in Table I.

Table I. Optical Adsorption Data of Phenols

	Wave-length of max. absorption (mμ)	Molar extinction coefficient
Phenol	269.5	1,520
o-Cresol	269.5	1,630
Thymol	273.5	1,980
o-Phenylphenol	282	3,970
p-Phenylphenol	258	18,800
Guaiacol	274.5	2,070
Eugenol	279.5	2,600
α-Naphthol	291.5	4,720
β-Naphthol	273.5	4,380

4) S. Oka, This Bulletin, **24**, 59 (1960).

RESULTS

Toxic effect of phenols The minimum phenol concentration required in order to inhibit the yeast growth for two days was determined. As seen in Table II, the toxic effect of phenols varies greatly with the kind of phenol, while the toxic effect is almost independent of the pH value of the medium.

TABLE II. TOXIC EFFECT OF PHENOLS ON YEAST

	Minimum inhibiting concentration (m-mol./kg-water)		
pH of medium	4.0	5.6	6.8
Phenol	22	22	24
o-Cresol	20	20	22
Thymol	1.0	1.0	1.1
o-Phenylphenol	1.2	1.2	1.3
p-Phenylphenol	0.3	0.3	0.3
Guaiacol	18	18	20
Eugenol	2.5	2.5	3.0
α-Naphthol	1.0	1.2	1.2
β-Naphthol	1·4	1.4	1.6

Koji extract (Ballg. 7°) containing phenols was inoculated with washed bakers' yeast cell in 0.0003%, and incubated for two days at 30°C.

Transfer of phenols to yeast cell The transfer of phenols from the medium to the yeast cell was examined. All of these phenols rapidly transfer to the yeast cell as shown in Table III. The transferred quantity is in a state of equilibrium with the concentration of the medium, and the equilibrium is independent of the pH value of the medium as shown in Table IV. In this case, the plot of the logarithm of the transferred quantity versus the logarithm of the equilibrium concentration gives a straight line as shown in Fig. 1. This relation is the same

TABLE III. CHANGE OF TRANSFER RATIO WITH SUSPENDING TIME

	Transfer ratio (kg-water/kg-cell)		
Suspending time (min.)	5	30	180
Phenol	1.65	1.63	1.66
o-Phenylphenol	21.0	21.0	20.8
α-Naphthol	19.4	19.6	19.6

The initial concentrations of phenol, *o*-phenylphenol and α-naphthol were 25, 2.0, and 1.4 m-mol./kg-water, respectively, and the proportions of the cell to the medium (pH 5.6) were 0.2, 0.05 and 0.05 kg/kg-water.

TABLE IV. INFLUENCE OF pH VALUE ON TRANSFER RATIO

	Transfer ratio (kg-water/kg-cell)		
pH of medium	3.5	5.0	7.0
o-Cresol	1.74	1.60	1.78
Thymol	25.7	26.8	25.9
β-Naphthol	17.2	16.8	17.0

The initial concentrations of *o*-creson, thymol and β-naphthol were 25, 0.7 and 1.4 m-mol./kg-water, respectively, and the proportions of the cell to the medium were 0.2, 0.05 and 0.05 kg/kg-water.

with that observed in cases of acid antiseptics and their esters[1~4], and is expressed by equation (1):

$$a = k(C_m)^n \qquad (1)$$

where a is the transferred quantity (m-mol./kg-cell), C_m the equilibrium concentration (m-mol./kg-water), k and n the constants of each phenol. The values of k and n are given in Table V. It is seen that the transfer ratio varies greatly with the kind of phenol.

The transferred quantity at the minimum inhibiting concentration was evaluated by substituting the minimum inhibiting concentration for C_m in equation (1) as given in Table VIII.

Dissolution of phenols in inner cell fluid The quantity of phenols dissolved in the inner cell fluid was evaluated from the influence of osmotic pressure on the transfer of phenols (cf. previous reports[1~3]). As shown in Table VI,

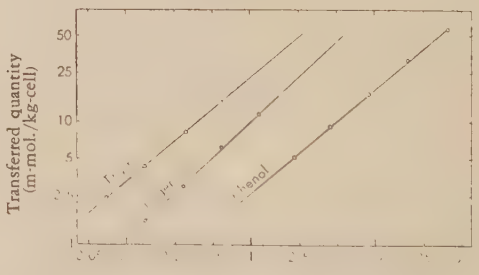

FIG. 1. Relation between Transferred Quantity and Equilibrium Concentration of Phenols.

1) Y.C.Su and K. Yamada, This Bulletin, **24**, 69 (1960).
2) Y.C.Su and K. Yamada, This Bulletin, **24**, 140 (1960).
3) W.W. Umbreit and I.C. Gunsalus, *J. Biol. Chem.*, **159**, 333 (1945).
4) P.A. Schaffer and M. Somogyi, *J. Biol. Chem.*, **100**, 695 (1933).

the dissolved quantity evaluated does not exceed 2 m-mol./kg-cell at the equilibrium concentration of 1 m-mol./kg-water. Judging from the fact that phenols can not be ionized in the inner cell fluid, the concentration of phenols in the inner cell fluid will be equal to that in the medium just as well as the cases of esters[2,3]. Thus, the dissolved quantity is represented by equation (2):

$$a_d = 0.65 C_m \qquad (2)$$

where a_d is the quantity dissolved in the inner cell fluid (m-mol./kg-cell), C_m the equilibrium concentration in the medium (m-mol./kg-water), and the value of 0.65 the water content of the cell (kg/kg-cell)[1].

The quantity dissolved at the minimum inhibiting concentration was evaluated by equation (2) as given in Table VIII.

Dissolution of phenols in lipid phase of cell In order to evaluate the concentration of these phenols transferred into the lipid phase of the cell, the partition of phenols between soy bean oil and water was examined. The plot of the logarithm of the concentration of phenols in the oil phase versus the logarithm of the equilibrium concentration in the water phase gives a straight line as shown in Fig. 2. This relation is expressed by equation (3) as well as the cases of esters[3]:

$$p = k'(C_m)^{n'} \qquad (3)$$

where p is the concentration of phenols in the oil phase (m-mol./kg-cell), C_m the equilibrium concentration in the water phase (m-mol./kg-water), and k' and n' the constants of each phenol. The values of k' and n' are given in Table VII.

In the previous report[3], it has been shown that the yeast lipid plays a similar role as soy bean oil as the solvent of lipophyl substances, and that the quantity of the yeast lipid relating to the partition is as great as 0.0156 kg/kg-cell[3]. Accordingly, the concentration of phenols in the yeast lipid at the minimum inhibiting concentration can be evaluated by equation (3), and the quantity dissolved in the yeast lipid is given by multiplying the concentration of the dissolved phenols by 0.0156 kg/kg-cell as given in Table VIII.

Table V. Transfer of Phenols from Medium to Yeast Cell

	k	n
Phenol	2.6	0.84
o-Cresol	2.2	0.92
Thymol	23	0.84
o-Phenylphenol	22	0.76
p-Phenylphenol	64	0.83
Guaiacol	1.7	0.99
Eugenol	9.8	0.94
α-Naphthol	19	0.81
β-Naphthol	16	0.83

The relation between the transferred quantity, a (m-mol./kg-cell), and the equilibrium concentration in the medium, C_m (m-mol./kg-water), is expressed by following Freundlich's adsorption isotherm.

$$a = k(C_m)^n$$

Table VI. Influence of Osmotic Pressure on Transfer

	Transferred quantity at 1 m-mol./kg-water of equil. conc. in med. (m-mol./kg-cell)		Ratio of dissolved quantity in cell to equil. conc. in med.
	Original buffer	2 mol./kg-water glycerin added	
Phenol	2.6	2.2	0.7
Thymol	23	22	1.7
α-Naphthol	19	18	1.7

The ratio in the last column of this table was evaluated by dividing the difference of transferred quantity in the original buffer and that in the buffer with glycerin by the relative decrease of the water content of the cell (0.6)[1].

Table VII. Partition of Phenols between Soy Bean Oil and Water

	k'	n'
Phenol	5.2	1.03
o-Cresol	5.2	1.03
Thymol	340	0.67
o-Phenylphenol	370	0.82
p-Phenylphenol	750	1.06
Guaiacol	13	0.79
Eugenol	84	0.68
α-Naphthol	240	1.06
β-Naphthol	160	1.15

The relation between the concentration of phenols in soy bean oil, p (m-mol./kg-oil), and the equilibrium concentration in water, C_m (m-mol./kg-water), is expressed by the following equation.

$$p = k'(C_m)^{n'}$$

TABLE VIII. DISTRIBUTION OF TRANSFERRED PHENOLS IN YEAST CELL AT MINIMUM INHIBITING CONCENTRATION

	Transferred quantity (m-mol./kg-cell)	Dissolved quantity in inner fluid (m-mol./kg-cell)	Dissolved quantity in lipid phase (m-mol./kg-cell)	Adsorbed quantity on solid phase (m-mol./kg-cell)
Phenol	35.0	14.3	2.0	18.7
o-Cresol	34.5	13.0	1.8	19.7
Thymol	23.0	0.7	5.3	17.0
o-Phenylphenol	25.3	0.8	6.7	17.8
p-Phenylphenol	22.5	0.2	3.2	19.1
Guaiacol	29.7	11.7	2.0	16.0
Eugenol	23.2	1.6	2.1	19.5
α-Naphthol	19.0	0.8	3.7	14.5
β-Naphthol	21.2	0.9	3.7	16.6

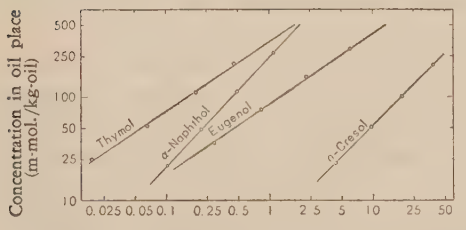

FIG. 2. Partition of Phenols between Soy Bean Oil and Water

DISCUSSION

By subtracting the quantities dissolved in the lipid phase and in the inner cell fluid from the corresponding total transferred quantity, the adsorbed quantity on the solid phase of the cell can be evaluated as shown in Table VIII. All of the adsorbed quantities thus evaluated are in fairly good agreement with each other under the same inhibiting condition, though the quantity dissolved in the lipid phase or in the inner cell fluid varies greatly according to the kind of phenols. Therefore, it is concluded that the quantity adsorbed on the solid phase of the cell is the limiting factor of the toxic effect of phenols and the toxic effect is independent of the quantity dissolved in the lipid phase or in the inner cell fluid.

The adsorbed quantity required to inhibit the yeast growth for two days was 14～19 m-mol./kg-cell in all cases of the phenols examined, and this value is in fairly good agreement with that obtained in cases of acid antiseptics and their esters[1~3]. From this fact, the inhibiting mechanism of these phenols was assumed to be the same as in cases of acid antiseptics and their esters, though the chemical form differs from each other

Acknowledgements The author wishes to express his sincere thanks to the late Emeritus Prof. R. Sasaki and Prof. Y. Sakurai of Tokyo University for their interest shown in this work. Thanks are also due to Dr. C. Hata, President of this Institute, for his support and encouragement in this work. The author is greatly indebted to Dr. M. Fujimaki, Dr. S. Okimasu, Dr. R. Nōmi and Mr. K. Shimizu for their kind advice.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 525~529, 1960]

Studies on L-Glutamic Acid Fermentation

Part III. Pilot Plant Scale Test of the Fermentative Production of L-Glutamic Acid by *Brevibacterium divaricatum* nov. sp.

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Received February 15, 1960

In this paper emphasis is placed on the utilization of fermentative production of L-glutamic acid in the pilot plant with formerly isolated *Brevibacterium divaricatum* nov. sp. using 500-L and 50-KL fermenters.

It was found that the yield of L-glutamic acid was higher than 45% in each case on the base of consumed glucose when the hydrolysates of Cassava starch (Tapioka flour) was employed as sugar source.

The greatest advantage observed in this experiment was the remarkable reduction in time required for fermentation in comparison with the fermentation time necessary for the laboratory method. This is a very favourable factor for the industrial preparation of L-glutamic acid.

In searching for the proper microorganism which may be used in industrial production, we have successfully isolated a new strain named *Brevibacterium divaricatum* nov. sp.[1] which has a high ability of accumulating L-glutamic acid.

The fundamental experiments concerning L-glutamic acid fermentation have been reported in the previous paper[2]. Hence only the experimental details using fermenters of 500 L and 50 KL in capacity, in the pilot plant will be described in this paper.

EXPERIMENTAL AND RESULTS

A. Fermentation by 500-L Fermenter.

1. Equipments and Analytical Methods.

The steam jacketed type fermenter made of SUS-7 stainless steel was used. The attached equipments were the urea feeding tank, defoamer tank, and an air filter.

Air was passed through the filter before it was sent into the fermenter by a sparger, 300 mm in diameter and having 100 holes of 2 mm diameter. The stirring was done with a pair of disk turbine type wings with the aid of two pairs of foam breaker (paddle type, 2 wings). The motor, totally fan-cooled-geard type, 5HP × 1/10, 440 V, 60 c/s, with a magnetic switch and push botton was used. The standard V-belts were driven to give an agitator speed of approximately 200 r.p.m. In

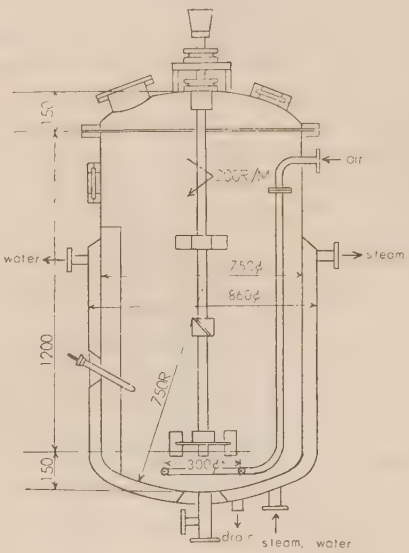

FIG. 1. 500 L Fermenter

1) Y.C.Su and K. Yamada, This Bulletin, **24**, 69 (1960).
2) Y.C.Su and K. Yamada, This Bulletin, **24**, 140 (1960).

TABLE I. MEDIUM COMPOSITION

Medium Composition	Seed Medium	Ferm. Medium
glucose	3.0%	10.0%[1]
urea	0.5	0.5[2]
K_2HPO_4	0.1	0.1
$MgSO_4 \cdot 7H_2O$	0.05	0.05
meat extract	0.3	0.2
peptone	0.3	—
10% bran extract	1.5 v/v	1.5v/v
pH	7.2	7.2~7.4

1) The sulfuric acid hydrolysate of Cassava starch, whose chemical components are shown in Table II, was used.
2) The initial concentration. A moderate amount of urea was added according to the change in pH during fermentation.

TABLE II. CHEMICAL ANALYSIS OF CASSAVA STARCH

Moisture	18.522%
Ash	0.144
Crude protein	1.319
Crude fibre	0.076
Crude fat	0.076
Nitrogen free extract	79.863

addition to these the fermenter had four baffle plates, the main specification of which is shown in Fig. 1.

For the quantitative analysis of L-glutamic acid, the manometric method using glutamic decarboxylase preparation obtained from *E. coli* najjar was adopted[3].

Glucose was determined by Somogyi-Shaffer-Hartman's method[4].

2. Culture Medium and Fermentation Conditions.

The seed and fermentation medium are shown in Table I.

Shaken cultivation of the seeds was carried out in a 500 ml flask containing 100 ml of medium at 30°C with shaking generated by a reciprocating shaker, 112 r.p.m., for 20 hours. Kd was kept at 5×10^{-6} g·mole of O_2/atm·min·ml.

After 20 hours' cultivation the residual sugar was found to be 0.6~0.8% and pH of the mixture around 5.0~5.4.

Two hundred liters of medium charged in a 500 L fermenter was sterilized at 110°C for 10 minutes. In order to start the fermentation, 300 ml of the above mentioned seed was inoculated to this sterilized medium. The inoculum size was about 0.15%.

Sterile air was introduced at the rate of 0.5~1.0 vol.

3) W.W. Umbreit and I.C. Gunsalus, *J. Biol. Chem.*, **159**, 333 (1945).
4) P.A. Schaffer and M. Somogyi, *J. Biol. Chem.*, **100**, 695 (1933).

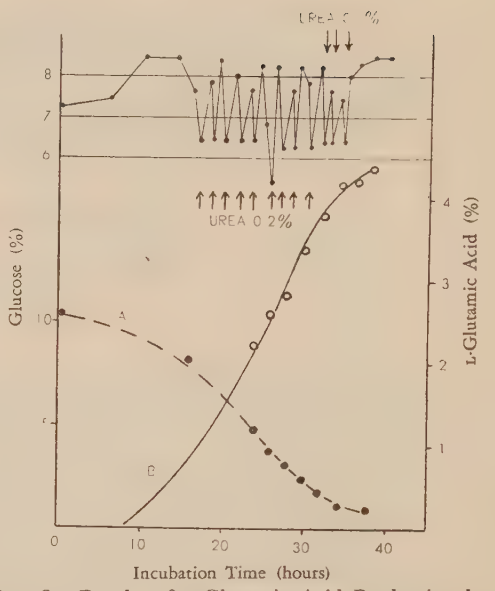

FIG. 2. Results of L-Glutamic Acid Production by the 500 L Fermenter (Test No. A-5).
A: Glucose
B: L-Glutamic acid

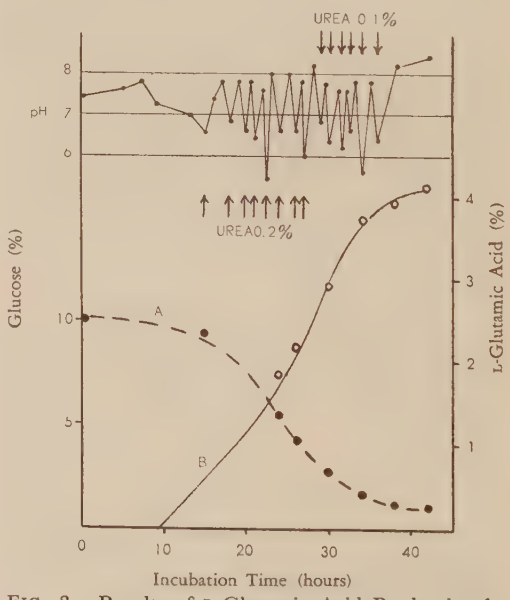

FIG. 3. Results of L-Glutamic Acid Production by the 500 L Fermenter (Test No. A-6).
A: Glucose
B: L-Glutamic acid

Table III. Material Balance between Main Raw Materials and L-Glutamic Acid Accumulated.

Test		No. A-5		No. A-6	
		Before Fermen.	After Fermen.	Before Fermen.	After Fermen.
Main Raw Materials	Glucose (kg)	20.6	2.32	20.12	2.10
	Urea (kg)	5.2	—	5.4	—
L-Glutamic Acid	Yield (kg)	—	8.72	—	8.2
	% Yield (Based on sugar source)	—	42.9	—	40.7
	% Yield (Based on consumed sugar)	—	47.7	—	45.5

per volume of the medium per minute. The stirring rate was 200 r.p.m. and the incubation temperature was maintained at 30~31.5°C.

Peanut oil was added as an antifoamer whenever the foam was violent.

3. Experimental Results.

The fermentation progresses of Tests No. A-5, and A-6 are shown in Fig. 2 and Fig. 3. The highest yield of L-glutamic acid was attained after 38 hours and 42 hours with respect to Nos. A-5 and A-6. Residual glucose was found to be 1.16% and 1.05%, respectively.

Material balance between main raw materials and formed L-glutamic acid is shown in Table III.

As is manifested the possibility to produce more than 45% of L-glutamic acid on the base of consumed hydrolysate of Cassava starch was observed.

B. Fermentation by 50 KL Fermenter.

1. Equipments and Analytical Methods.

The fermenter was made of soft steel with two attached tanks, urea feeding tank (capacity, 3000 L) and defoamer tank (capacity, 1000 L). Predescribed 500 L fermenter was used as seed cultivation tank. The stirring was accomplished by two pairs of 6 wings disk turbine with the aid of two pairs of foam breaker (paddlle type, 2 wings) and two of perforated plates.

The motor, vertical totally-enclosed fan-colled type (100 HP × 8 P, 3450 V, 60 c/s) with starting compensator, equiped with standard V-belts was driven to give an agitator speed of approximately 100 r.p.m. Dimensions of major parts are shown in Fig. 4.

The stainless steel cooling coil was immersed in fermenter so that it would absorb lots of heat generated during fermentation.

Air line was so arranged that the air from the air compressor (1.8~2.0 kg/cm² gauge) was guided through after cooler in order to remove water before they were driven through air filter and sent into fermenter by sparger. The ring type sparger (800 mm diameter) consists of upper and lower sections. Each section has 2000 holes, 3 mm in diameter.

The exhaust was forced through cyclone before it was driven out to atmosphere.

An orifice meter was used to measure amount of air introduced. To measure the pH glass electrode pH meter was applied. Temperature read from the dial thermometer were recorded.

2. Culture Medium and Fermentation Conditions.

Medium composition was identical with the one shown in Table I. Procedures from seed cultutre to main fermentation were as follows;

Agar slant culture ⟶ 500 ml flask shaking culture ⟶ 500 L fermenter ⟶ 50 KL fermenter.

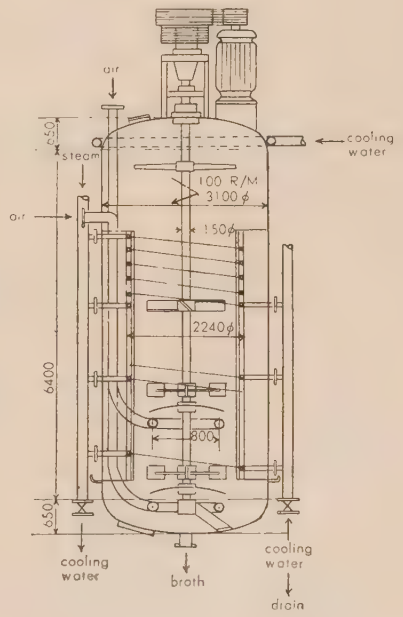

Fig. 4. 50 KL Fermenter

Three hundred milliliters of seed culture (a loopful of seed was inoculated/100 ml medium in 500 ml flask) was inoculated 500 L fermenter where its volume was increased to 300 L. It was then inoculated to the main fermentative medium. Inoculum size was about 1.0~1.5%

Sterile air was introduced at the rate of 0.25~1.0 vol. per volume of the medium per minute. The stirring rate was 100 r.p.m. and the incubation temperature was maintained at 32~34°C.

The peanut oil was added as antifoamer whenever the foam was violet. Inside pressure of the fermenter was kept at 0.2 kg/cm² gauge.

3. Experimental Results.

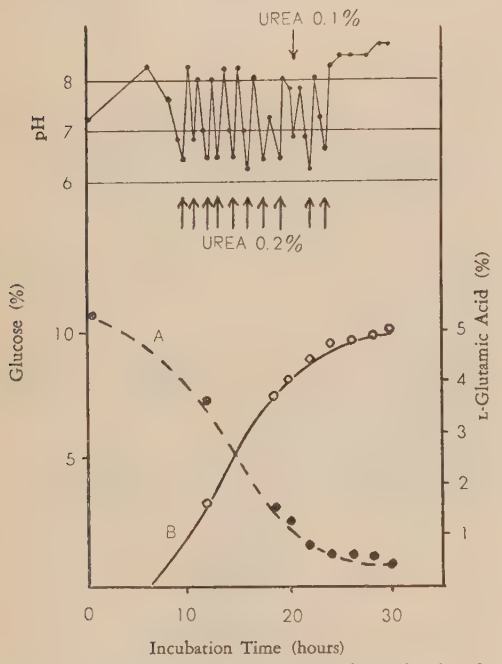

FIG. 5. Results of L-Glutamic Acid Production by the 50 KL Fermenter (Test No. C-7).
A: Glucose
B: L-Glutamic acid

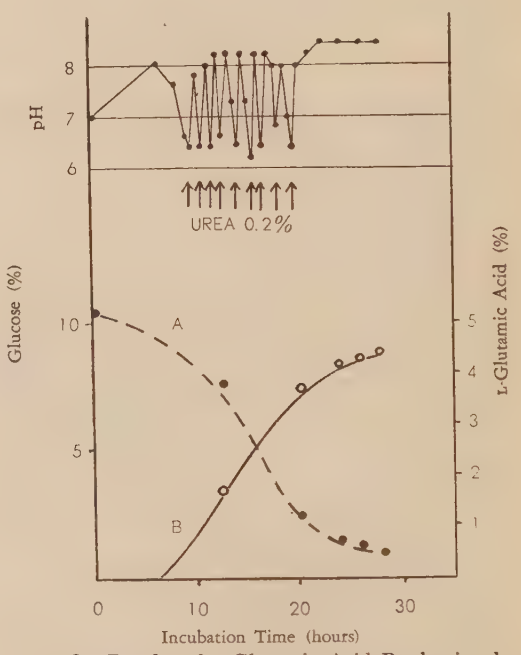

FIG. 6. Results of L-Glutamic Acid Production by the 50 KL Fermenter (Test No. C-9).
A: Glucose
B: L-Glutamic acid

TABLE IV. MATERIAL BALANCE BETWEEN MAIN RAW MATERIALS AND L-GLUTAMIC ACID.

Test		No. C-7		No. C-9	
		Before Fermen.	After Fermen.	Before Fermen.	After Fermen.
Main Raw Materials[1,2]	Glucose (kg)	2349.8	217.8	2574	240
	Urea (kg)	573.5	—	576.5	—
L-Glutamic Acid	Yield (kg)	—	1126.1	—	1087.5
	% Yield (Based on sugar source)	—	47.9	—	42.2
	% Yield (Based on consumed sugar)	—	52.8	—	46.5

1) The amount of raw materials needed for seed culture were also included.
2) Hydrolysate of Cassava starch was used.

In Fig. 5, and Fig. 6 the progresses of Test No. C-7 (charged liquid 22 KL, meat extract 0.15%) and Test No. C-9 (charged liquid 25 KL, meat extract 0.2%) were shown respectively.

Though similar progresses were observed as was in the experiment using 500 L fermenter, it was found that the incubation time was greatly reduced by increasing inoculum size and maintaining pH correctly at the range of 6~8. This is no doubt the noteworthy advantage in industrial preparation.

Material balance between main raw materials and L-glutamic acid formed is shown in Table IV.

Both the amounts of charged liquid and meat extract, the growth factor, were different in Test No. C-7 and No. C-9. Results showed the former test, No. C-7, gave the better yield.

In L-glutamic acid fermentation with *Micrococcus glutamicus*, Kinoshita[5] pointed out the regulative function of biotin, the growth factor, and oxygen, whose partial pressure changed by condition of aeration and stirring intensity, to control the accumulating ability of L-glutamic acid.

This is interesting because the phenomenon has resemblance to what was found in this experiment.

5) S. Kinoshita, *J. Fermtn. Tech.* (*Japan*), **37**, 547 (1959).

Note

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 530~531, 1960]

Isolation of L-Leucyl-L-Proline Anhydride from the Culture Filtrate of *Streptomyces* sp. S-580

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Received November 25, 1959

Previously L-leucyl-L-proline anhydride had been isolated from hog adrenal cortex extract[1] and, in 1951, was also isolated by J. L. Johnson et al.[2] from the culture filtrates of an unidentified *Streptomyces* species, a streptomycin-producing strain of *Streptomyces griseus* and *Aspergillus fumigatus* H-3 which had produced an inhibitor of the action of *S. aureus* phage. As subsequently described, the same substance has been isolated from the culture filtrate of *Streptomyces* sp. S-580 from which L-prolyl-L-valine anhydride, a new diletopiperazine, was isolated[3,4].

Culture of *St.* sp. S-580 and extraction of the crude syrup were carried out through the same method previously reported[5]. After repeated extraction of the crude syrup with a sufficient volume of hot ethyl ether and evaporation of the solvent under reduced pressure, pale yellow crude crystals appeared. Through recrystallization from acetone, colorless hexagonal plates appeared as shown in Fig. 1. This substance is a neutral colorless hexagonal plate (from acetone), m.p. 158~161°C, $[\alpha]_D^{23°C} = -143.7$ (in ethanol), soluble in acetone, alcohols, benzene, chloroform and water, and very slightly soluble in *n*-hexane and petroleum ether. Analytical data of this substance was as follows: *Anal.* Calcd. for $C_{11}H_{18}O_2N_2$: C, 62.83: H, 8.63: N, 13.32: mol. wt., 210.27, Found: C, 62.86: 62.94: H, 8.45; 8.56: N. 13.17; 13.04: mol. wt. (detr. by the micro Rast method dissolving in camphor), 221.27. Therefore, this substance was proved to have the empirical formula: $C_{11}H_{18}O_2N_2$. The UV-spectrum of this substance in methanol showed a strong end-absorption, however, not significant in the wavelength range of 211~400 mμ. The infra-red spectrum of this substance in Nujol mull is shown in Fig. 2. Both the ninhydrin and the biuret reactions on this substance were found to be negative. However, hydrolysis in hydrochloric acid gave two amino acids, leucine and proline and these have the L-configulation because the activity of D-amino acid oxidase towards them was negative when determined by the manometric

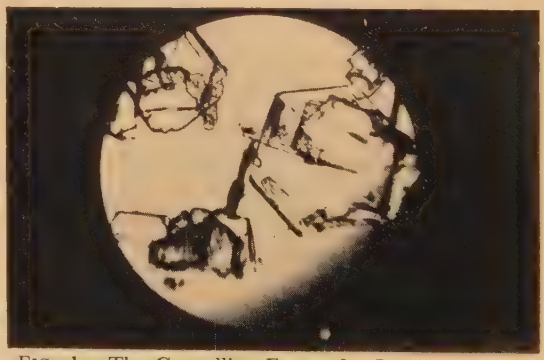

FIG. 1. The Crystalline Form of L-Leucyl-L-proline Anhydride Isolated. (magnification × 80)

Presented at the Annual Meeting of the Agricultural Chemical Society of Japan held in Kyoto, May 3, 1958.

* Present address, Research Laboratory, Meiji Seika Kaisha, LTD.

1) O. Wintersteiner and J.J. Pfiffner, *J.B.C.*, **111**, 599 (1935).
2) J.L. Johnson et al., *J.A.C.S.*, **73**, 2946 (1951).
3) Y. Koaze, This Bulletin, **21**, 197 (1957).
4) Y. Koaze, *ibid.*, **22**, 98 (1958).
5) Y. Koaze, *ibid.*, **22**, 91 (1958).

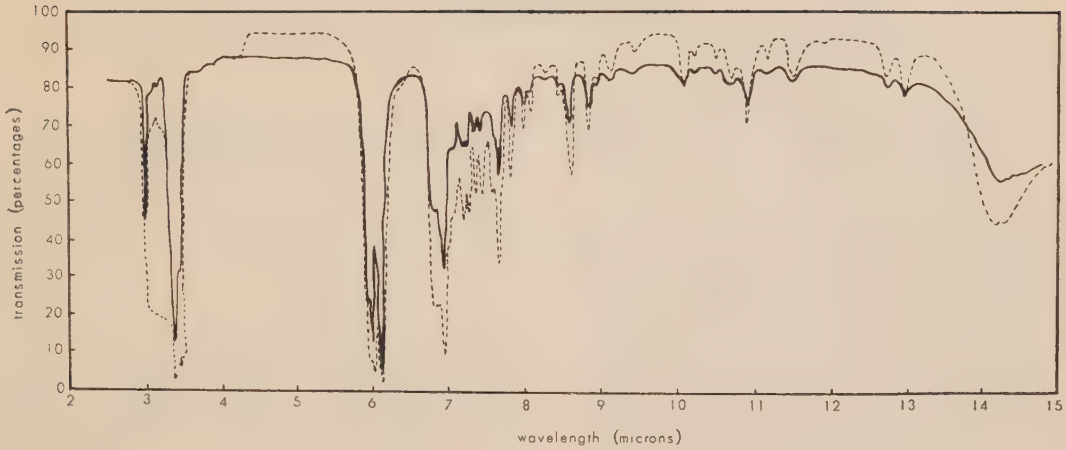

FIG. 2. Comparison between the Infra-red Spectrum of this Substance and Synthesized L-Leucyl-L-proline Anhydride (diketopiperazine) in Nujol Mull.

method. Furthermore through the microbioassay, the hydrolyzate of 1 mol. of this substance consisted of 1 mol. of L-leucine and L-proline.

On the other hand, L-leucyl-L-proline anhydride was synthesized from N-carbobenzoxy-L-proline and L-leucine methyl ester through the same procedure as previously described[4]. The infra-red spectrum of the synthetic sample in Nujol mull is shown in Fig. 2 and a mixed melting point with the isolated sample gave no depression. Therefore, the isolated sample was clearly identical with the synthesized sample, L-leucyl-L-proline anhydride.

The author wishes to express his sincere thanks to Prof. K. Sakaguchi, Univ. of Tokyo for his constant guidance throughout this work, and also to Prof. Y. Sumiki, Univ. of Tokyo, for his kind advice and suggestion. Thanks are also due to Mr. I. Matsumoto, Banyu Pharmaceutical Co. Ltd., for his kind gift of phosgene, and further extended to Mr. J. Kirimura, Sericultural Experiment Station, Ministry of Agriculture and Forestry, for his help in the microbioassay. The elemental analysis and infra-red spectrum were carried out at this department.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 532, 1960]

Blätteraldehyd: Eine einfache Synthese und Konfiguration

Sehr geehrter Herr Schriftleiter:

Blätteraldehyd wurde von T. Curtius und H. Franzen[1] aus Hainbuchenblättern erstmals isoliert. Seither besteht dieses Name, da das Aldehyd zusammen mit dem Blätteralkohol im Pflanzenreich verbreitet vorkommt und den eigentümlichen Geruch nach grünen Blätter zeigt. Einige Untersuchungen über das Blätteraldehyd sind seit Mitteilung[1] veröffenlicht worden[2~4]. Dennoch kann man in diesen Berichten über Konfiguration und einfache, ergiebige Synthese nichts finden. Besonders war die Konfiguration des Blätteraldehyds als trans-Form nur hypothetisch angenommen worden, da die Stabilität der cis-$\alpha\beta$-ungesättigen Aldehyde theoretisch sehr gering zu sein scheint[5]. Die Untersuchungen des Blätteraldehyds ist reizvoll, da er aus riechstoffchemischen und pflanzenphysiologischen Gesichtspunkten wichtig ist. So ist er Insektenlockstoff[6] sowie mögliches Bruchstück des Sexual-lockstoffs[7], *n*-Hexadeka-dien-(10,12)-ol-(1), und mag ein Faktor[8] sein, der für die Schutzfarbe von "Papilio xuthus L" entscheidend ist.

Über vier Stufen (II, III und IV: unbekannte Substanzen) wurde in bedeutend Ausbeute besser als bisher dargestellt.

$$\underset{\text{(I)}}{CH_3CH_2CH_2COCl} \xrightarrow[AlCl_3]{CH\equiv CH} \underset{\text{(II)}}{CH_3CH_2CH_2COCH=CHCl} \xrightarrow{NaOH/CH_3OH} \underset{\text{(III)}}{CH_3CH_2CH_2COCH_2CH(OCH_3)_2} \xrightarrow{LiAlH_4}$$

$$\underset{\text{(IV)}}{CH_3CH_2CH_2CH(OH)CH_2CH(OCH_3)_2} \xrightarrow{10\%H_2SO_4}$$

$$CH_3CH_2CH_2-\underset{H}{\overset{H}{C}}=\underset{H}{C}-CHO$$
(V)

Durch Einleiten von Acetylen in Buttersäurechlorid (I) (mit $AlCl_3$) wurde Propyl-β-chlorvinylketon (II) in sehr guter Ausbeute erhalten. Sdp. 75°/31mm, n_D^{12} 1.4688, Ausb. 86.3%, IR-Spektrum: –COCH=CH–Absorption bei 6.3μ, –CH=CHCl bei 11.3μ. Bei der Behandlung von (II) mit NaOH/CH_3OH-lösung wurde β-Oxy-*n*-hexanal-dimethylacetal (III) wurde, (IV) aus (III) durch Hydrierung mit $LiAlH_4$ gewonnen. Sdp. 110°/24mm, n_D^{10} 1.4405, Ausb. 75.8%. Nach der Wasserdampfdestillation mit 10% H_2SO_4 des β-Oxy-*n*-hexanal-dimethylacetals (IV) siedete das gewonnene 2-trans-Hexen-1-al bei 144~46°. n_D^{20} 1.4499, Ausb. 93% (50% überalles). IR-Spektrum: $-\underset{H}{\overset{H}{C}}=\underset{H}{C}-$Absorption bei 10.3$\mu$, –C=C–CHO bei 6.1$\mu$, –CHO bei 3.5$\mu$. 2,4-Dinitrophenylhydrazon (VI): Schmp. 146~47°, $C_{12}H_{14}O_4N_4$ Ber. C 51.79 H 5.03 Gef. C 51.85 H 5.13

Aus der Identität des IR-Spektrums von authentischem natürlichen Blätteraldehyd-2,4-dinitrophenylhydrazon (VII)[9] mit (VI) sowie keiner Schmelzpunktserniedrigung beim Mischen von (VI) mit (VII), ist es aus dem experimentalen Beweis, ohne Zweifel zufolgen, daß die Konfiguration des natürlichen Blätteraldehyds die trans-Form ist.

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Eingegangen am 5, März 1960

1) T. Curtius und H. Franzen, *Ann.*, **390**, 89 (1912).
2) ebenda, **404**, 93 (1914).
3, 9) S. Takei, Y. Sakato und M. Ohno, *Bull. Inst. Phys. Chem. (Tokyo)*, **13**, 128 (1934).
4) S. Takei, Y. Sakato, M. Ohno und Y. Kroiwa, *Bull. Agr. Chem. Soc. (Japan)*, **14**, 709 (1938).
5) R. Raphael und F. Sondheimer, *J. Chem. Soc.*, **1951**, 2693.
6) T. Watanabe, *Nature*, **182**, 325 (1958).
7) A. Butenandt, R. Beckmann, D. Stann und E. Hecker, *Naturforschg.*, **14**, 283 (1959).
8) T. Hidaka, "*KAGAKU*", **27-2**, 93 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 533~534, 1960]

The Production of D-Araboascorbic Acid by a Mold

Sir:

This is the first report on the production of D-araboascorbic acid by a mold. It was very rare case that D-araboascorbic acid has been found in biological materials or products. Isherwood[1,2] et al. have found it in cress seedling in D-altrono-γ-lactone solution and in the rat injected with D-mannono-γ-lactone.

The strain belonging to the *Penicillium* isolated from soil produced D-araboascorbic acid from D-glucose, D-gluconic acid and sucrose. The media contained 2% D-glucose, nitrogen sources and minerals.

The *Penicillium* was incubated stationarily for 5~7 days at 27°C. The broth showed strong reducing power to 2,6-dichlorphenol-indophenol and the R_F-value on the paper chromatograph by actonitrile coincided with that of synthetic D-araboascorbic acid. The contents of D-araboascorbic acid in the broth were up to 2mg per ml.

After impurities were eliminated from the broth by Ba-acetate and charcoals, the supernatant was run through the anion resin IR-4B column[3]. The 1N HCl solution was used for elution. The effluent was evaporated in vacuo at 25~30°C under the aeration of CO_2. Alcohol was added and evaporated to remove the excess of water. After the syrup was dried in vacuum desiccator for 3~5 days, the white crystals were separated from the mother liqour by cold acetone. The crystals were washed with cold acetone and recrystallized from methanol dioxane mixture.

The chemical properties of this crystal are following; m.p. 172°C (uncorr.)

Anal.: Calculated for $C_6H_8O_6$

C 40.92%, H 4.58%.

Found: C 41.27%, H 4.66%.

$[\alpha]_D^{26.5°} = -23$ (1% in water)

The infra-red spectrum of this crystal and the synthetic D-araboascorbic acid of the General Chemicals Co. (U.S.A.) are showed in Fig. 1 (a,b,c,)

The 2,4-dinitrophenyl-hydrazone of this compound was prepared from the resin effluent by the method of Jackel[4]. The chemical properties of the osazone are following;

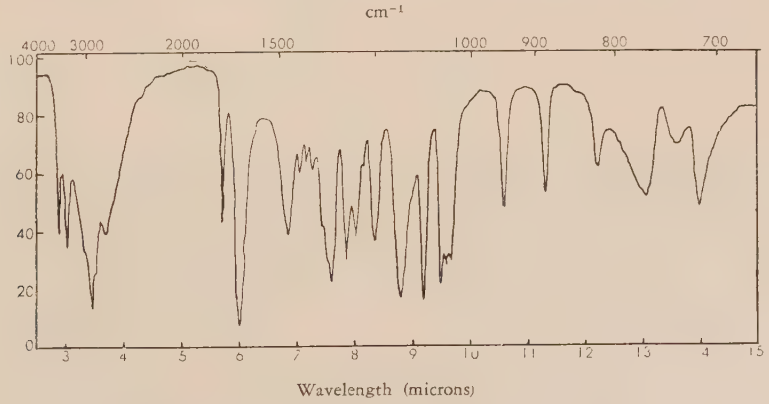

FIG. 1-a. Infra-red Spectrum of Reducing Substance

m.p. 238~242°C

Anal.: Calculated for $C_{18}H_{14}O_{12}N_8$

C 40.44%, H 2.64%, N 20.97%.

1) F.A. Isherwood, Y.T. Chen and L.W. Mapson, *Nature*, **171**, 348 (1953).

2) F.A. Isherwood, Y.T. Chen and L.W. Mapson, *Bioch. J.*, **56**, 1 (1954).

3) H.H. Horowitz, A.P. Doershuk and C.G. King, *J. Biol. Chem.*, **189**, 193 (1952).

4) S.S. Jackel, E.H. Mosbach and C.G. King, *Arch. Biochem.*, **31**, 442 (1951).

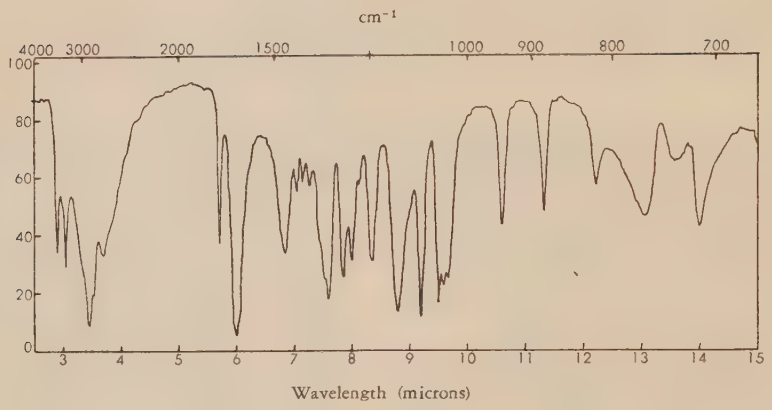

FIG. 1-b. Infra-red Spectrum of Syn. D-Araboascorbic Acid

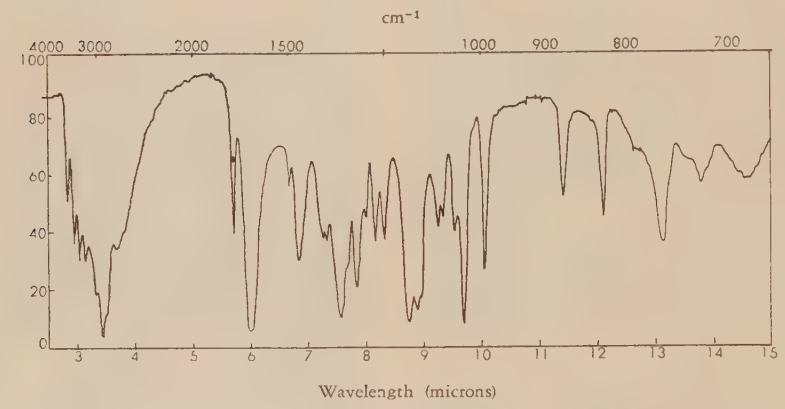

FIG. 1-c. Infra-red Spectrum of L-Ascorbic Acid

Found: C 41.55%, H 2.84%, N 21.15%.

The L-ascorbic acid productions by molds have been reported[5,6], however, the contents in the broth was only about 0.03mg per ml at the highest level and it was produced from not only glucose, but also glycerin.

In our experiment, D-araboascorbic acid was produced from only D-glucose, D-gluconic acid and sucrose; and not from glycerin. These facts are very important to analyse the mechanism of its formation.

The authors wish to express their sincere thanks to Prof. T. Asai and Prof. K. Yamada, University of Tokyo, Dr. H. Okazaki, Sankyo Co. Ltd., for their kind advices.

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Received April 23, 1960

5) M. Geiger-Huber and H. Galli, *Helv. Chim, Acta*, **28**, 248 (1945).
6) K.S. Stantry, *Nature*, **179**, 44 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 535~536, 1960]

Formation of 5-Dehydrofructose by Members of *Acetobacter*

Sir:

In the course of study on the utilization of fructose by ketogenic bacteria, it was found that a new strong reducing substance, 5-dehydrofructose (or 5-ketofructose), was formed from fructose by a wide variety of members of *Acetobacter*.

This substance was formed in a medium containing 5~10% of fructose and 0.3~0.5% of yeast extract, and was isolated in a crystalline form (Fig. 1).

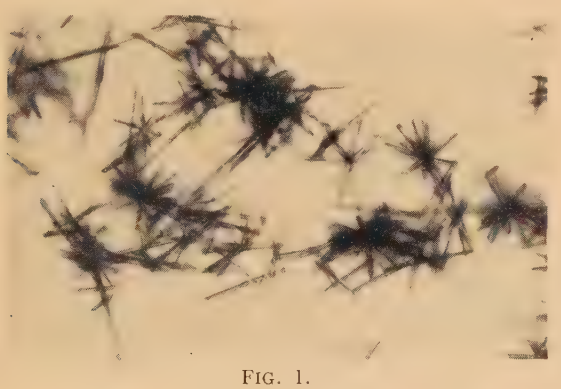

FIG. 1.

Among a number of cultures tested, some cultures belonging to *Acetobacter suboxydans*, *A. oxydans*, *A. gluconicum*, *A. melanogenus*, *A. roseus*, *Gluconobacter cerinus* and *G. roseus* were found to form this substance, but some cultures belonging to the same species showed no productivity.

The new substance was recovered from the fermented liquor by such a process as follows: the fermented liquor was clarified by centrifuge, deionized with ion exchange resins, decolorized with charcoal, concentrated in vacuo to a heavy syrup, and then extracted with ethanol. The ethanol extract was concentrated in vacuo, and the crystal formed was washed with chilled ethanol, then with ether, and dried over calcium chloride under reduced pressure.

The crystal was colorless, small prisms with sweet taste; not deliquescent; melted at 162~163°C; freely soluble in water, glycerol and glycols; slightly soluble in ethanol and ethyl acetate; insoluble in ether and benzene. It reduced Fehling's solution rapidly at room temperature and its reducing power was about 110% of glucose.

The molecular weight of this substance was determined to be 178.9~186.0 by a freezing point depression method. This result support the molecular equation of $C_6H_{10}O_6$ (178.14).

Anal. Calcd. for $C_6H_{10}O_6$: C, 40.45; H, 5.65%. Found: C, 40.89, 40.71; H, 6.05, 5.58%.

$[\alpha]_D^{18.5} = -90.0°$ (c=2.50, water). No mutarotation was observed.

This substance reacted rapidly with phenylhydrazine at room temperature forming pale yellow plates. It melted at 137~138° C and its elementary analysis coincided with that of bisphenylhydrazone.

Anal. Calcd. for $C_{18}H_{22}N_4O_4$: C, 60.32; H, 6.19; N, 15.63%. Found: C, 60.34, 59.56; H, 5.68, 6.44; N, 16.44, 15.54%.

Periodate has been known to decompose one mol. of ketose into (i) one mol. glycolic acid, three mols. of formic acid and one mol. of formaldehyde (main reaction), or (ii) one mol. of glyoxylic acid, two mols. of formic acid and two mols. of formaldehyde (subsidiary reaction). It is also known that the formation of glycolic acid is characteristic of compounds containing CH_2OH–CO– radical in their molecules.

In the reaction mixture of this substance with periodate, the presence of glycolic and glyoxylic acid was detected chromatographically, and the definite identity of glycolic acid was also confirmed by the formation of its *p*-bromphenacyl ester, m.p. 141°C.

Quantitative analysis on the same reaction mixture showed that this substance yielded just twice as much glycolic and glyoxylic acid as

equimolar fructose.

These results, together with the fact that this substance was stable to bromine oxidation, suggest the presence of two CH_2OH–CO– radicals in its molecule.

The existence of two carbonyls in the molecule of this substance was also proved by the hydroxylamine hydrochloride titration method.

By the stepwise reduction with borohydride, the new substance was reduced to two ketoses which have the same R_F values as those of fructose and sorbose, and not the same as those of tagatose and psicose. This is another fact to support the presumption that this substance to be 5-dehydrofructose.

All these experimental results, mentioned above, indicate this new substance to be 5-dehydrofructose.

It is of particular interest that this is the first instance of the direct oxidation of ketose by Bertrand's rule.

The biochemical significance of this substance is under study now, and will be published elsewhere.

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Received June 1, 1960

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 536~537, 1960]

The Structure of Neocycasin C formed by Transglycosylation with Cycad Emulsin*

(Studies on Some New Azoxyglycosides of *Cycas revoluta* Thunb. Part VI)

Sir:

The authors reported in the previous paper[1] that by a transglycosylation with cycad emulsin, β-glucosyloxyazoxymethane, cycasin[2], is transformed into its β-laminaribiosyl derivative, neocycasin A[3], and afterwards also into a new higher oligoside, neocycasin C. The last was inferred to be produced from neocycasin A, though could not be investigated in detail because of its poor yield.

Here is presented the proof for the above inference, and also the structure of neocycasin C. A mixture, 0.8 ml, containing neocycasin A (0.075 M in final concentration), acetate buffer (pH 5.6) and the enzyme (0.7 mg) was incubated at 30° C and the products were investigated at intervals by paper chromatography. In the early stage of the reaction, glucose, cycasin and laminaribiose as decomposition products appeared. The appearance of neocycasin C was recognized in six hours, and it increased gradually with time. Although some faint spots of oligosaccharides and neocycasin B, gentiobioside[4], were detected in later, neocycasin C remained to be the major transfer product in forty-eight

* This work was supported in part by the grant to K. Nishida in Aid for Scientific Research from the Ministry of Education, and was presented at the meeting of the Agricultural Chemical Society of Japan held in Tokyo, April 6, 1960.

1) T. Nagahama, K. Nishida and T. Numata, This Bulletin, **24**, 322 (1960).

2) K. Nishida, A. Kobayashi and T. Nagahama, This Bulletin, **19**, 77 (1955).

3) K. Nishida, A. Kobayashi, T. Nagahama and T. Numata, This Bulletin, **23**, 460 (1959).

4) T. Nagahama, T. Numata and K. Nishida, This Bulletin, **23**, 556 (1959).

hours.

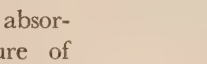
In order to isolate these products, the reaction mixture of twenty folded scale was incubated for twenty-four hours, and was chromatographed on carbon column and then preparatively on paper sheets. The following reaction products, including unaffected neocycasin A, were separated in chromatographically pure state: glucose, gentiobiose (1.1 mg), laminaribiose (50.6 mg), cycasin (45.9 mg), neocycasin B (1.3 mg) and C (9.1 mg), and unknown oligosaccharides, 'x' (1.1 mg), 'y' (1.9 mg) and 'z' (1.5 mg). The last four were examined further.

Neocycasin C displayed in its ultraviolet absorption spectrum the characteristic feature of azoxyglycosides ($\lambda_{max.}=216$ mμ, $\lambda_{inflex.}=275$ mμ).

In its partial hydrolysate (0.2 N H_2SO_4, 100°C, 1 hr.), the spots of glucose, laminaribiose and the two corresponding to 'y' and 'z' as sugars, and of cycasin, neocycasin A and spots A_x as glycosides were recognized. On the one hand, the sugar component of neocycasin C was liberated intact after the decomposition of its aglycone by reduction with zinc-acetic acid. This sugar travelled in the same R_F value as 'z', and these two were partially hydrolysed into the same products, glucose, laminaribiose and the corresponding one to 'y'. Since 'y' was hydrolysed to give glucose and laminaribiose, it must be laminaritriose. Thus, the sugar component of neocycasin C and 'z' are proved to be laminaritetraose. The above conclusion on tri- and tetra-ose was further ascertained by a regularity of their log $[R_F/(1-R_F)]$ values with those of glucose and laminaribiose.

Consequently, it is concluded that neocycasin C is β-laminaritetraosyloxyazoxymethane, and the spot A_x detected in the hydrolysate of this tetraoside should be laminaritrioside. The products by these procedures were formulated as follows:

	R_F*
neocycasin C	.39
spot A_x (trioside)	.49
neocycasin A	.59
cycasin	.68



CH_3–N(→O) : N–CH_2–O-$\underline{\beta^1}$G-$\underline{\beta^1}$G-$\underline{\beta^1}$G-$\underline{\beta^1}$G

glucose	.62
laminari-biose	.51
laminari-triose ('y')	.40
laminari-tetraose ('z')	.30

*using BuOH-pyridine-H_2O (6 : 4 : 3) as solvent for multiple ascending (3 runs)

The sugar 'x' was inferred to be glucobiose from the result of its hydrolysis, but not identified. In the above enzymatic reaction triose was detected, but not trioside. Including these problems, the transferring specificity of cycad emulsin which catalyses preferential formation of β-1,3 glucosidic linkage should be further investigated.

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Received May 9, 1960

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 538~540, 1960]

The Iodine Affinity of Amylopectin

Sir:

Ito and Yoshida[1] have reported previously a method for determining the iodine affinity of starchy materials by means of amperometric titration with iodine, in wnich a rotating platinum electrode is used. We have examined several factors affecting the determination and have partly revised the procedure. Detailed description of the proposed method will be published elsewhere[2]. The method gives exactly the same values of iodine affinity (grams of iodine consumed by 100 g material) as those determined by a dead-stop method of Larson, Gilles and Jenness[3] as well as by the potentiometric method of Lansky, Kooi and Schoch[4].

In applying the method to potato amylose and amylopectin, we are able to confirm that amylopectin fractions give no iodine affinity in amperometric titration. When artificial mixtures of potato amylose and amylopectin are titrated, curves shown in Fig. 1 are obtained. Amounts of iodine consumed in complex formation with starchy materials are proportional to the amylose content. Curves in Fig. 2 are obtained in the titration of potato amylose in varying amounts (5~25 mg/50 ml). The iodine affinity (18.29± 0.02) calculated from each curves has agreed very well with each other.

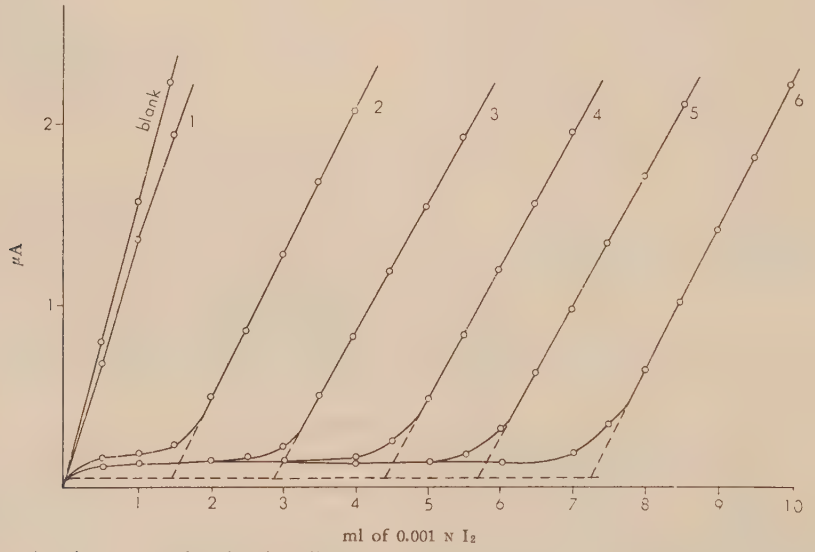

FIG. 1. Amperometric Titration Curves of Mixture of Potato Amylose and Amylopectin

1. Amylopectin 5 mg
2. Amylopectin 4 mg Amylose 1 mg
3. Amylopectin 3 mg Amylose 2 mg
4. Amylopectin 2 mg Amylose 3 mg
5. Amylopectin 1 mg Amylose 4 mg
6. Amylose 5 mg

However, the titration of amylopectin in varying amounts (10~50 mg/50 ml) gives curves shown in Fig. 3. Curves in Fig. 3 did not have any horizontol parts shown in Figs. 1 and 2,

1) M. Ito and S. Yoshida, This Bulletin, **23**, 34 (1959).

2) T. Kobayashi and S. Yoshida, *Denpun Kōgyōgaku Kaishi* (*J. Technol. Soc. Starch*), in the press.

3) B. L. Larson, K. A. Gilles and R. Jenness, *Anal. Chem.*, **25**, 802 (1953).

4) S. Lansky, M. Kooi and T. J. Schoch, *J. Am. Chem. Soc.*, **71**, 4066 (1949).

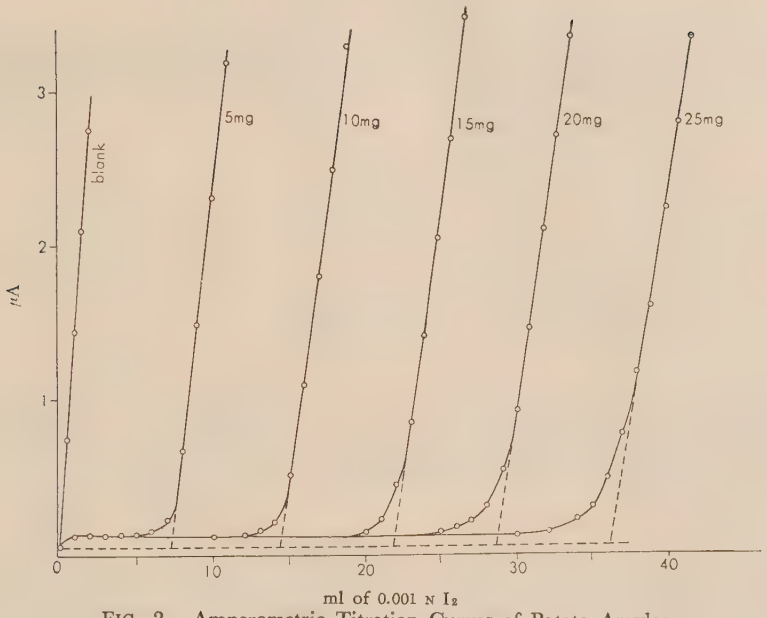

FIG. 2. Amperometric Titration Curves of Potato Amylose

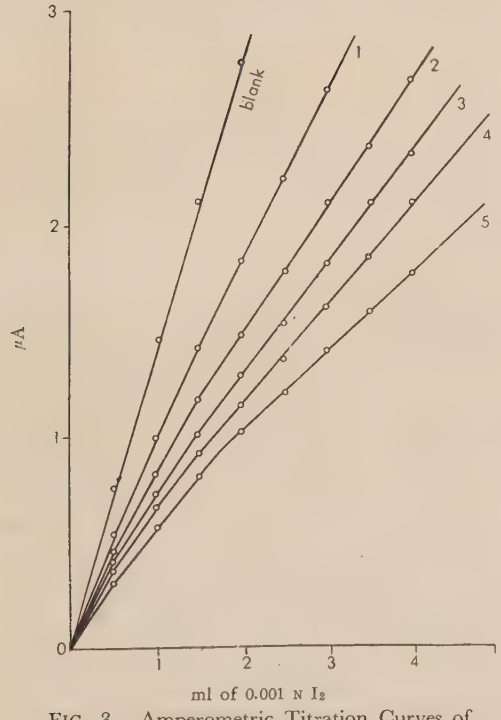

FIG. 3. Amperometric Titration Curves of Potato Amylopectin

curve 1: 10 mg 2: 20 mg 3: 30 mg
4: 40 mg 5: 50 mg

and no iodine affinity can be calculated therefrom. The fact indicates that amylopectin would be stained by iodine in a manner rather different from the formation of helical complex as postulated on amylose.

Very low but definitely positive values have been reported for iodine affinities of amylopectin in potentiometric method[4], and Larson et al.[3] have also found a small sorption of iodine with an amperometric titration based on dead-stop method. But the former result is probably ascribed to the inability of the potentiometric method to discriminate strictly the iodine consumed in complex formation from mere adsorption, and the latter result is due to a small amount of amylose contaminated in amylopectin preparations as suggested by Larson et al.[3].

It is not certain at present whether the amylopectin does not form any helical complex with iodine at all or does form a very loose complex. But it may be suggested that another mechanism than helical complex formation would be possible for starch-iodine coloration. Apparently the nature of interaction of amylo-

pectin and iodine requires more detailed investigation, and further works are now in progress.

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Received June 15, 1960

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 540~542, 1960]

Synthese and Configurational Elucidation of Rotenoids

Part XVIII. The Total Synthesis of the Natural Rotenone

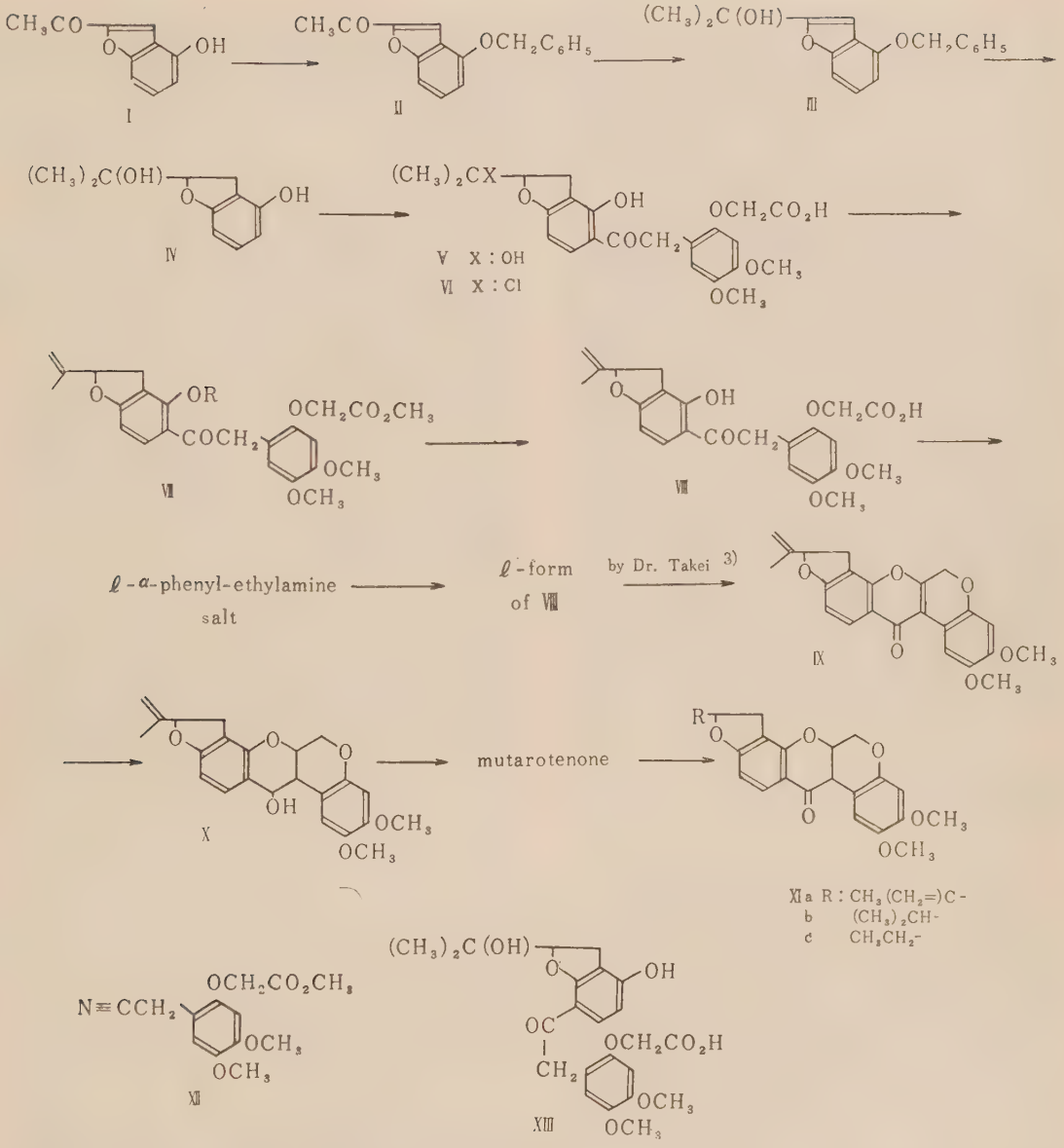
Sir:

The chemical structure (XIa) of rotenone, an insecticidal principle in derris root, was determined in 1932 by three groups headed by Butenandt[1], LaForge[2], and Takei[3]. Its partial synthesis from derrisic acid (*l*-form of VIII), one of the degradation products of rotenone, was accomplished by Miyano and Matsui[4,5] in 1958, while dihydrorotenone (XIb)[6,7] and *dl*-nor-dihydrorotenone (VIc)[8] were totally synthesized by the same authors recently.

In this communication we wish to report the total synthesis of the natural *l*-rotenone which is the first paper to our knowledge. Starting from 2-acetyl-4-hydroxycoumarone (I) which had been prepared in 6 steps from resorcinol[12~15] by other workers, we synthesized *dl*-2-(α-hydroxy-isopropyl)-4-hydroxycoumaran (*dl*-hydroxydihydrotubanol) (IV) through the benzyl ether (II)[7], following Grignard's reaction to 2-(α-hydroxyisopropyl)-4-benzyloxy-coumarone (III)[7~9] and finally hydrogenation over Raney nickel.

Now a Hoesch' reaction of IV with a nitrile[11] (XII) under the specified condition followed by saponification resulted in a mixture which was consisted of *dl*-hydroxydihydroderrisic acid (V) (main product), *dl*-chlorodihydroderrisic acid (VI) and an isomer (XIII) of V, among those only XIII could be isolated in crystalline state (recrystallized from methanol, m.p. 153~155°, calculated for $C_{23}H_{26}O_9 \cdot CH_3OH$: C 60.24, H 6.32, found: C 60.18, H 6.88. IR-Spectrum in Nujol paste: carboxylic C=O 1725 cm^{-1}, ketonic C=O 1660 cm^{-1}). The other two acids were separated by chromatography. IR-Spectrum of VI in chloroform (carboxylic C=O broad, near 1760 cm^{-1}; ketonic C=O 1645 cm^{-1}) is almost equal to that of dihydroderrisic acid in all regions. IR-Spectrum of carbonyl region of V in chloroform (carboxylic C=O broad, near 1760 cm^{-1}; ketonic C=O 1643 cm^{-1}) is also similar to that of VI. The methylester of V (oil)

1) A. Butenandt and W. McCartney: *Ann.*, **494**, 17 (1932).
2) F. B. LaForge and H. L. Haller: *J. Am. Chem. Soc.*, **54**, 810 (1932).
3) S. Takei, S. Miyajima and M. Ohno: *Ber.*, **65**, 1041 (1932).
4) M. Miyano and M. Matsui: This Bulletin, **22**, 128 (1958).
5) M. Miyano and M. Matsui. *Chem. Ber.*, **91**, 2044 (1958).
6) M. Matsui and M. Miyano: *Proc. Japan Academy*, **35**, 175 (1959).
7) M. Miyano and M. Matsui: *Chem. Ber.*, **92**, 2487 (1959).
8) M. Miyano and M. Matsui: *ibid.*, **93**, 54 (1960).
9) M. Miyano and M. Matsui: This Bulletin, **24**, 218 (1960).
10) M. Miyana and M. Matsui. *Chem. Ber.*, **93**, in press (1960).
11) A. Robertson: *J. Chem. Soc.* (London), **1933**, 1163.
12) A. A. Snamshurin: *Zhur. Obshei Khim.*, **16**, 1877 (1946); *Chem. Abstr.*, **41**, 6237 (1947).
13) R. Robinson and R. C. Shah, *J. Chem. Soc.* (London), **1934**, 1946.
14) R. C. Shah and M. C. Laiwalla, *ibid.*, **1938**, 1828.
15) Organic Syntheses, Coll. Vol. 2, 557 (1948).



prepared with diazomethane within a restricted time afforded on treatment with phosphorous bromide in cold pyridine an oily dehydrated compound (VII, R=P(OH)$_2$) accompanying other unidentified products which was then converted in the free *dl*-derrisic acid (VIII) (recrystallized from ethanol, m. p. 158~160°). IR-Spectrum of the synthetic *dl*-VIII in chloroform solution (C=CH$_2$ 908 cm^{-1}; carboxylic C=O broad, near 1760 cm^{-1}, ketonic C=O 1645 cm^{-1}) is completely identical with that of the *l*-derrisic acid (m. p. 151°) of natural origin, while they are not equal in Nujol paste. Therefore our synthetic sample is clearly the

racemic form of derrisic acid. The analytical value was consistent with the theoretical figure; calculated for $C_{23}H_{24}O_8$; C 64.47, H 5.65; found (sample 0.229 mg, CO_2 0.525 mg, H_2O 0.168 mg): C 62.56, H 8.21. Further we demonstrated indirectly that *l*-α-phenyl-ethylamine forms the ethanol-insoluble salt [m. p. 162~3°, $[\alpha]_D^{22}$—49.0 (c=2.72, chloroform) calculated for $C_{31}H_{35}O_8N$: C 67.74, H 6.42, N 2.55; found: C 67.58, H 6.78, N 2.60] only wih *l*-derrisic acid (but not with its optical antipode, and *l*-derrisic acid (m.p. 151°, recrystallized from methanol, calculated for $C_{23}H_{24}O_8 \cdot 1/2CH_3OH$: C 63.50, H 5.90; found: C 63.34, H 6.19) can be recovered from the salt by the ordinary method.

A partial synthesis of *l*-rotenone, the natural modification of the possible 8 stereoisomers of XIa, from *l*-derrisic acid was already described in literatures[4,5]. Shortly outlined, *l*-derrisic acid was cyclized to dehydrorotenone (IX) by Takei[3] with boiling acetic anhydride in the presence of sodium acetate which was then converted into rotenol (X) by sodium borohydride in warm dioxane[4,5]. The latter on Oppenauer oxidation afforded mutarotenone, a stereoisomer of the natural rotenone which converted into the natural modification by thermal isomerization[4,5]. The total scheme is again given here which consists of a sequence of 11 steps from the known compound (I) or 17 steps from the commercially available resorcinol.

Since the rotenone was derived by other workers[16~19] through iso-dihydrorotenone to dihydrodeguelin, this report also means a total synthesis of dihydrodeguelin.

We will publish the more detailed results in other place.

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Received August 17, 1960

16) H. L. Haller and F. B. LaForge: *J. Am. Chem. Soc.*, **53**, 4460 (1931).
17) S. Takei, S. Miyajima and M. Ohno: *Ber.*, **66**, 479 (1933).
18) H. L. Haller: *J. Am. chem. Soc.*, **53**, 733 (1931).
19) S. Takei, S. Miyajima and M. Ohno: *Ber.*, **66**, 1826 (1933).

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 34, No. 5 (1960)

Studies on the Changes of Milk Proteins by *Penicillium roqueforti*. Part II. Proteolysis by Enzyme Preparation and Effects of Lactic Acid Bacteria. (p. *375*～*378*)

By Tsuneaki IMAMURA
(*Department of Agriculture, Okayama University*)

In order to obtain some basic knowledges on the casein breakdown by *P. roqueforti* and other starter organisms, changes of tyrosine levels in substrates by enzyme preparations were determined. Maximum proteolysis was observed at pH 5.5 in the ranges of 5.0～8.0 and 42° in that of 3～50°. Proteolytic activity of cell extract from fungus mat increased with molds' growth until the growth had reached to maximum point at 7th day from beginning, but thereafter, decrease of activity of cell extract and increase of cell free medium were observed owing to the protease had become to extracellular enzyme. In such extracellular state, the activity was rapidly lossed at high temperature (25°) during few days. As ripening of Roquefort cheese has been performed at 4.5～10°, it was considered from the above results that the mold's growth and its protease production were slowly but the activity was maintained for a long time. Starter organisms such as *Str. lactis*, *Str. cremoris*, and *Lact. bulgaricus* had also proteolytic action, but their activities were neglegible weak in comparison with that of *P. roqueforti* and accerelation effect on tyrosine production by cell extract was observed. Accordingly, their predominant effect on the proteolytic action was probably to accelerate the molds' growth by improvements of lactose consumption and acidity of medium.

Studies on the Changes of Milk Proteins by *Penicillium roqueforti*. Part III. Effects of Rennet. (p. *379*～*383*)

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The effects of rennet was studied with the changes of tyrosine level, electrophoretic patterns and paperchromatograms of free amino acids. The enzyme preparation used were molds' cell extract (CE), Hansens' rennet tablet, Grublers' pepsin, and crystalline rennin. The tyrosine production by CE was accelerated by the cooperative action of rennet. This fact was also observed by pepsin and not by rennin. When the mixture of pepsin and rennin at various ratio were added to casein substrate with CE, tyrosine production was more accerelated at higher levels of pepsin. From the above results, the effects of rennet was considered to be occured by pepsin in it and not by rennin. Then, the relation of tyrosine production by CE and degree of rennet action was studied. From the results of this experiments and electrophoretic patterns following conclusion was induced: CEs' peptidase action was comparatively weak and the decomposition of peptides from casein was supplemented by pepsins' peptidase action. This was supported by the paparchromatograms of free amino acids liberated by these enzyme preparations. Lysine and arginie wich were terminal groups of α-casein were liberated by rennin.

Studies on the Changes of Milk Fat by *Penicillium roqueforti*. Part II. Lipolysis by Enzyme Preparation. (p. *383*～*387*)

By Tsuneaki IMAMURA
(*Department of Agriculture, Okayama University*)

The level of bat decomposition was determined by titration of fatty acids which licerated by cell extract and cell-free medium of *P. roqueforti* and maximum decomposition was observed at pH 7.0 and at 30～35°. The degree of lipolysis during incubation increased with the molds' growth, but the specific activity was lowered in stationary phase. Such lipase action was not observed in molds' enzyme preparation form normal Czapeks' solution in which did not contain oil or fat, but there was remarkable lipase activity in bread incubated with mold as Roquefort cheese starter. The amount of volatile acid liberated by cell extract was equivalent to 1/4～1/3 part of the titratable value of total free acid and this ratio by cell-free medium was more than a half. From such specificities of each enzyme preparation, presence of two different type of lipase was assumed and it was ascertained with two centrifugal

fractions, i.e., supernatant and sediment. The op-pH of lipase action of these two fractions were 6.5 and 7.5, especially. The activities of cell extract and cell-free medium were lowered by addition of more than 3% of NaCl.

Studies on the Changes of Milk Fat by *Penicillium roqueforti*. Part III. Accumulation of Volatile Fatty Acids and Effects of NaCl. (p. *387*～*391*)

By Tsuneaki IMAMURA

(*Department of Agriculture, Okayama University*)

The lack of oxygen supply was not sufficient to explain the accumulation of volatile fatty acids in cheese, since it had been found that *P. roqueforti* could grow under such anaerobic condition as interior of Roquefort cheese. Then, the effects of NaCl on the mold growth, volatile fatty acids consumption, and their accumulation were investigated. The growth of mold was slightly accerelated and the amount of volatile fatty acids, especially insoluble acids, accumulated in milk was increased by addition of NaCl less than the extent in cheese, i.e., 4%. But the consumption of volatile fatty acids was lowered with the increase of MW. and the consumption level of acetic acid became to 1/2, that of butyric to 1/3, of caproic to 1/5, and of caprylic to 1/6 by 4% of NaCl addition. Due to the remarkable decrease of consumption, contrary to the slightly effect of lipase activity as previous report, the accumulation of volatile fatty acids was increased, probably. The pH of milk was lowered and acidity was increased only by addition of NaCl or KCl. This fact was assumed to be caused by loss of buffer activity or liberation of hydrogen ion which was adsorbed to protein particles. Accumulation of Volatile fatty acid in skimmilk was not observed, but the decomposition of residual milk fat rather contribued to the volatile fatty acids production than lactose or amino acids.

Studies on the Organism Producing Isopropanol from Acetone. Part IV. Enzymological Studies on the Oxido-Reduction of *Lactobacillus brevis* var. *hofuensis*. (1) (p. *391*～*397*)

By Kazuo HOSHINO, Kiyoshi UTAKAWA and Kenzo WATABE

(*Kyowa Fermentation Industry Co. Ltd.*)

In this paper present authors have made an attempt to clarify the mechanism of isopropanol formation by *Lactobacillus brevis* var. *hofuensis*. Primarily, it was visualized that the intact cells, dried cells and crude extract of this organism could reduce acetone to isopropanol in the presence of glucose as hydrogen donor. Secondarily, it was demonstrated that the acetone-isopropanol system requires TPN as cenzyme and is capable of coupling with TPN-linked G-6-P and 6-PG dehydrogenases presented in the cell-free enzyme preparation of this organism. From the enzymic studies on the acetone-isopropanol system following specific properties were observed: (1) redoxpotential of this system shows approximately −0.3 volts by means of color change of oxidation-reduction dyes, (2) isopropanol is more reactive than ethanol as substrate, while in TPNH oxidation ethanol-acetaldehyde system is much reactive.

Polarographic Studies on Storage of Meats. Part IX. Influence of Gamma-Ray Irradiation on Organic Acids and Free Amino Acids of Beef. (p. *397*～*403*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

In the present paper, we have carried out the experiment on the change in organic acids and free amino acids during 20°C storage for 52 days by using the irradiated beef vacuum-packaged into polyethylene-cellophane bag under the same conditions as in a previous experiment.

The results of the experiment mentioned above are as follows:

(1) In each case of the irradiated beefs before the 20°C storage, there is an increase in the lactic acid content with increasing dosages of the γ-ray, but in case of dosages more than 240×10^4r, there is a decrease in its content.

In each case of the irradiated beefs before the 20°C storage, there is a decrease in the free creatinine and creatine content with increasing dosages of the γ-ray. But in the dose level of 800×10^4r, there is not a remarkable decrease in those.

When each amino acid content of the irradiated beef is determined immediately after irradiation, a prominent change in each content due to the different γ-ray dosages, is not seen.

(2) In each case of the beefs irradiated less than 148×10^4r, there is a decrease in its lactic acid content with increasing days of 20°C storage. In case of dosages more than 200×10^4r, there is a decrease in the lactic acid content up to storage time of 21 days, but beyond this period, there is an increase with storage time.

In each case of the irradiated beefs, there is a decrease in the free creatine and the third substance content with

increasing days of 20°C storage, but there is not a decrease in the creatinine content during 20°C storage.

In the case of the irradiated beefs after the 20°C storage, there is a characteristic change in each amino acid content with increasing days of 20°C storage and dosages of the γ-ray.

(3) The third substance mentioned above is not yet definitely known by us. It is observed during the irradiation of γ-ray. It is not produced during 20°C storage and in the beefs which are non-irradiated and irradiated in the dose level of 800×10^4r.

(4) The lactic acid or each amino acid content which is determined from the irradiated beef after 20°C storage, is less. This result indicates that the protein wave from the extract of the beef is little affected by those.

Studies on the Utilization of D-Glucosamine. Part I. A Synthesis of L-Serine. (p. *404~405*)

By Hiroyoshi KUZUHARA and Masanao MATSUI

(*Faculty of Agriculture, Tokyo University*)

The preparation of optically active amino-compounds (having the desired configuration) from D-glucosamine, using the original assym. carbon, were studied. In this paper was described newer synthesis of L-serine in moderate yield from D-glucosamine through N-benzoyl-D-glucosamine, N-benzoyl-D-glucosaminol and N-benzoyl-L-serine aldehyde. An analogous synthesis was already reported by K. Tsurumi and S. Yamada, although in poor yield[1].

1) K. Tsurumi and S. Yamada, *Tohoku J. Expl. Med.*, **62**, 329 (1955).

Studies on the Aerobic Sugar Metabolism of Yeast. Part VI. Gluconic Acid Production by Shaking Culture. (II) (p. *406~408*)

By Shoichi TAKAO

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To obtain a higher yield of gluconic acid conditions suitable for inoculating *Candida* sp., which was found to be the best gluconic acid producing yeast by shaking culture, were examined.

As a result, a large amount of gluconic acid was produced in a very short cultivation period when cells pre-cultured on a shaking machine for one day were inoculated. The best conditions were; sugar concentration of pre-culture 2%; volume of inoculum 15ml; NH_4Cl concentration of mother culture 0.045%. The yeast gave gluconic acid in a yield of 100% of the employed glucose when cultivated by shaking method for 24 hrs.

Studies on DL-Forming Lactic Acid Bacteria. Part IV. Asparagine as an Essential Growth Factor of *Lactobacillus saké*. (p. *409~411*)

Akira ŌBAYASHI (*Faculty of Agriculture, Kagoshima University*) and Kakuo KITAHARA (*Institute of Applied Microbiology, University of Tokyo*)

Since all strains of *Lactobacillus saké* are unable to grow in a semisynthetic medium which generally are used for lactic acid bacteria, some factor had been supposed to be essential for growth of this species.

From 'Mikuni-peptone' one of these factors was purified and identified as asparagine.

Essential amino acids for the growth of this species were also investigated.

Reaction of Furan Derivatives with Ammonia. Part VI. On the Reaction of 2-Furyl-Arylketones with Ammonia. (p. *411~416*)

By Hiroshi SUGISAWA, Hironari SUGIYAMA and Kiyoshi ASO

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Previous works have shown that 2-alkyl-3-hydroxy-pyridines and 2-alkyl-pyrrylketones were obtained by reacting 2-furyl-alkylketones with ammonia. This investigation was subjected to a similar reaction in the hope that 2-aryl-3-hydroxy-pyridines and 2-aryl-pyrrylketones might be synthesized from 2-furyl-arylketones.

There was obtained corresponding 2-aryl-3-hydroxy-pyridines (II) and 2-aryl-pyrrylketones (III) by reacting 2-furyl-arylketones (I), as shown in following, with ammonia at 180° for 20 hrs.

R:	(I)	(II)	(III)
Phenyl	m.p. 43~4°	m.p. 202°	m.p. 78~9°
Tolyl	m.p. 41~2°	m.p. 200°	m.p. 120°
3-Methyl-4-methoxy-phenyl	m.p. 81~2°	m.p. 181~2° Picrate 205°	m.p. 139°
3-Methyl-4-hydroxy-phenyl	.....	m.p. 241~2°	.....

On Protone Resonance of Sodium-Higher-Acylcarboxylates-Water System. (p. *416~420*)

By Syun NOGUCHI

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It is well known that many physico-chemical studies on sodium salts of higher fatty acids were already published, but no appeares in accord with nuclear magnetic resonance of the compounds.

Now, the present attempts have been made for the studies on the proton absorption resonance of sodium-higher acylcarboxylates-water systems. A proton resonance absorption curve is assumed as a superposition of two lines. They are a very narrow and a broad. The former is due to the motional or amorphous component in the sample and the latter to the rigid or crystalline component. The ratio of the rigid protons to the total ($[W]$) is suggested to be decreased linearly comparable to the increase of water content (w) and to be assumed that there is a simple relation between the both as follows: $[W]=S_0\frac{100-w}{100}$

where S_0 is the value of the $[W]$ at anhydrous state ($w=0$) and S_0 may be corresponded to the degree of crystallinity, and α is a constant showing the degree for the effect of water on the structure of the salts.

The results were seen in S_0 about 0.95~0.99 and in α far smaller than unit (<1) for the salts of saturated acylcarboxylates except caprate and on the contrary, in S_0 about 0.5~0.6 and in $\alpha>1$ for the unsaturated ones respectively. Consequently, the confirmation may be briefly summarized, as follows. The crystallinity degree of the salts of saturated fatty acids indicated much larger than that of the unsaturated ones. A part of water containing in the saturated compounds were seen to be fixed on the rigid components and on the other hand, a part of the rigid component in the unsaturated ones seemed to be charged to the motional component by adding water.

Determination of Hydrated Water in Sodium-Higher-Acylcarboxylate-Water Systems by Measurement of Dielectric Constant. (p. *420~423*)

By Shun NOGUCHI

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The principle is a well-known fact that the dielectric constant of the hydrated water in substance is considerably smaller than that of the unhydrated (at radio-wave region) and consequently, if the preparation contains a small amount of free water, the marked increase of the dielectric constant may be seen.

Recently, the author has attempted to elucidate the measurement of dielectric constant for determining the hydrated water in sodium-acylcarboxylate-water systems.

After repeated experiments, it was found that sodium saturated carboxylate (C_8~C_{18}) and sodium-elaidate have a definite hydrated water (about 3%) and that of unsaturated (*cis* form) has not. The results seem to be in good agreement with our previous works on the vapor pressure, electric conductivity and nuclear magnetic resonance of soap-water systems.

These findings lead to conclusion, whether soap has hydration or not may depend on the configuration and crystabillity of molecule.

Researches on Ergot Fungus. Part XXXVI. On the Mode of the Conversion of Elymoclavine to Agroclavine. (p. *424~427*)

By Saburō YAMATODANI and Matazō ABE

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It was found that elymoclavine yielded lysergol and a basic substance on treatment with hot sodium butylate. The basic substance, $C_{16}H_{16}N_2$, mp. 244~245° (uncorr. decomp.), $[\alpha]_D^{20}=+407°$ (in $CHCl_3$), $[\alpha]_D^{20}=+463°$ (in pyridine), was obtained in an excellent yield also by the treatment of lysergol itself with hot sodium butylate. Moreover, this basic substance exclusively yielded festuclavine on catalytic hydrogenation, whereas, it yielded the same substance together with agroclavine, pyroclavine, costaclavine and lysergine [6,8-dimethyl-ergolene-(9)] on reduction wish sodium and *n*-butanol. These facts indicated that the basic substance corresponded to 6-methyl, 8-methylene-ergolene-(9), for which the authors have adopted the designation lysergene. From these results, it was evident that the conversion of elymoclavine to agroclavine by reduction with sodium and *n*-butanol was performed through lysergol and lysergene.

It was presumed that agroclavine must be produced also in the living cells through the same process as described above.

This paper was communicated in This Bulletin, **21**, 200 (1957).

Researches on Ergot Fungus. Part XXXVII. Relationship between the Ergot Alkaloids Triseclavine and Isosetoclavine. (p. *427~429*)

By Matazō ABE, Saburō YAMATODANI, Tōgo YAMANO and Mitsugi KUSUMOTO

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It was found that there are two kinds of strains in the *Agropyrum*-type ergot fungus, one of which produces far more triseclavine than isosetoclavine, the other producing far more the latter than the former, while the

strains belonging to the *Elymus*-type fungus produce triseclavine almost exclusively. The facts indicated that triseclavine as well as isosetoclavine present in nature or in saprophytic culture was produced by the action of fungi. The chemical and physical examinations, especially that of the dissociation constants, led the authors to believe that triseclavine and isosetoclavine are isomers differing from each other in the arrangement of the substituent at C^8. From the result, it seemed pertinent to rename isosetoclavine isotriseclavine.

This paper was communicated in This Bulletin, **22**, 59 (1958).

Studies on the Utilization of *Streptomyces griseus* Protease. Part I. Utilization of *St. griseus* Protease for Miso-manufacture. (p. *430~433*)

By Masao NOMOTO (*The Institute of Physical and Chemical Research*), Fusanosuke MASUKO and Tsutomu OHASHI (*Masuko Food Co., Ltd.*).

As previously reported by the authors, a new protease produced by *Streptomyces griseus* has a very broad substrate-specificity and is able to cleave almost all kinds of peptide-bond of protein. Using this superior hydrolysing activity of the protease for miso-manufacture, a product of good qualities was obtained by a new manufacturing process, as follows: After being steamed at 120° in an autoclave and cooled to 40° under air-tight condition, 60 kg of soy bean is mixed with 27.3 g of the protease and kept at 40° for 12 hours under aseptic condition. After the reaction, the partially digested soy beans are well mixed with 25 kg of sodium chloride and 36.5 kg of koji. The mixture is kept at 33° for 24 days and then at room temperature for 2 weeks or more. The resulting product (i.e., miso) produced by the aboue method was found to be much better in its taste and gloss and also higher in its amino acid concentration than that produced by the conventional method.

Études sur la fermentation acétono-butylique. IV. Caractères taxonomiques de *Clostridium*, souche N 314 et sa position dans la systématique. (p. *434~439*)

par Seinosuke SUGAMA et Shinji DOI

(*Faculté d'Agronomie de l'Université de Nagoya, Anjô, Japon*)

Depuis McCoy et Peterson, on pense que la négligeable production de gaz et d'acide à partir de pectine par le "butyl organism" est due à une impureté de cette dernière. Les caractères et surtout la propriété pectinolytique de N 314 ont été étudiés en comparison de ceux de 11 souches butyriques et acétono-butyliques dont 3 souches originelles de Weizmann: ATCC 4259, ATCC 824 et N 179. L'ensemble des propriétés de N 314 coincide avec celui des souches de Weizmann. Malgré que la pectine en eau peptonée ne soit que légèrement fermentée par ces germes, les gels de pectine en milieu de Kaiser sont liquéfiés par les mêmes organismes: en 3 jours après l'ensemencement, N 314 fait diminuer 80% et 100% respectifs de la viscosité des gels de pectine et de pectate. La quantité de pectine décomposée est proportionnelle à la dépression de la viscosité. Parce que ces germes fermentent fortement le D-galacturonate, ils équipent évidemment la polygalacturonidase liquéfiant les gels de pectine presque sans les saccharifier. En ce qui concerne la pectine, *C. acetobutylicum* déterminé par l'ancienne méthode, avait impliqué la possibilité de porter les éléments hétérogènes et ces derniers doivent être appartenus à *C. omnivorum*. Pour les accepter dans la position de *C. omnivorum*, les caractères plus communs de ce dernier doivent être suivants: *Clostridum* incolore, pectinolytique, glucidolytique et protéolytique (gélatine liquéfiée; lait coagulé, puis hydrolysé).

Studies on the Effects of Some Physical Conditions on the Submerged Mold Culture. Part IV. Effects of the Culture Fluid Viscosity on the Mycelial Forms of Molds. (p. *440~442*)

By Jōji TAKAHASHI, Yoshiyasu KOSANO and Koichi YAMADA.

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

In the shaking culture of molds, it is known that the mycelium may grow into the homogeneous and filamentous suspensions (pulp type growth), or develop into the form of globose colony (pellet type growth).

Among many physical conditions which were considered to affect the type of mycelial growth, in this paper, the effects of culture fluid viscosity on the mycelial forms of *Asp. niger* NRRL-337 and *Pen. chrysogenum* Q-176, were investigated respectively.

In the culture of *Asp. niger*, the flocculence of germinating conidia which was observed in the culture fluid of lower viscosity (1~5 C.P.), could not be found in that of higher viscosity (20~50 C.P.). Therefore, the pellet formation which originated from the flocculence of conidia, was prevented by the addition of suitable viscous materials to the culture fluid, and the more viscous the medium became, the more filamentous mycelium was obtained.

In the culture of *Pen. chrysogenum* in which the flocculence of conidia could not be found, little effect of medium viscosity on the mycelial forms was observed.

Studies on the Effects of Some Physical Conditions on the Submerged Mold Culture. Part V. Effects of the Culture Fluid Viscosity on the Growth and the Amylase Production of *Asp. niger* NRRL-337.

(p. *442～447*)

By Jōji TAKAHASHI, Haruo MACHIDA and Kōichi YAMADA.

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The mycelial form of *Asp. niger* NRRL-337 is remarkably affected by the culture fluid viscosity, that is, the more viscous the medium becomes, the more flamentous and more homogeneous form of mycelium is obtained. Therefore, it is expected that the mycelial growth and the amylase production are influenced by the medium viscosity.

From this point of view, in this paper, following results are reported.

(1) When the mycelium developed into pellet form in the culture fluid of lower viscosity (1～5 C.P.), the yield of dry mycelium per unit sugar consumed was very high in the early stage of growth, but it decreased remarkably in the later stage of growth. On the contrary, when the culture of pulp form was obtained in the medium of higher viscosity (20～50 C.P.), the yield of mycelium per unit sugar was nearly constant throughout the every stage of growth, and the more viscous the fluid became, the lower yield per unit sugar consumed was obtained.

(2) In the culture of pellet form, the accumulation of the endocellular amylase increased rapidly over the end of growth phase, and decreased markedly in the later stage of cultivation. Less accumulation of endocellular amylase was found in the culture of pulp form, but it remained nearly constant after the utilizing sugar had been eliminated.

(3) The amount of exocellular amylase produced by unit weight of pulp from mycelium, throughout the every phase of cultivation, was larger than that of pellet form. The maximum yield of amylase per unit weight of mycelium, consequently, increased accordicg as the medium viscosity increased, that is, the more filamentous the mycelium became, the better production of amylase was obtained.

Studies on the Bitter Testing Peptides produced by Proteinases. Part II. Chemical Properties of the Bitter Tasting Peptides by "Neutral Proteinase" of *Bacillus subtilis*.

(p. *448～452*)

By Kazuhiro ICHIKAWA, Takehiko YAMAMOTO, Akira NISHIO and Juichiro FUKUMOTO

(*Faculty of Science, Osaka City University*)

One of the bitter tasting peptides which are produced at the digestion of casein by bacterial neutral proteinase was purified according to the method reported previously and the amino acids constituent, N-terminal amino acid, the behavior of the peptide in relation to the bitterness towards acid, alkali and various proteinases were investigated. The peptide was consisted of leucine, phenylalanine, methionine, proline, tyrosine, tryptophan, threonine, arginine, glutamic acid, glycine, serine and alanine. No sugars nor phosphorus were sound in the peptide. The N-terminal amino acid of the peptide was found to be leucine. Heating the peptide in the presence of dilute acid or alkali resulted in easy loss of the bitterness, although there occured only slight hydrolysis in the peptide links. On the other hand, treating with proteinases which were obtained from *Rhizopus chenensis*, *Rhizopus niveus* and *Streptomyces griseus* caused no change in the bitterness in spite of bringing considerable extents of hydrolysis on the peptide.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 34, No. 6 (1960)

Nutritional Studies on Fluorine. Part II. The Effect of Calcium on Fluorine Metabolism in Rats. (p. *453~456*)

By Susumu FUJIE
(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

1. The effect of calcium on the metabolism of fluorine, mottled teeth, and the hardness or strongness of teeth and bones of rats administered 2 mg of fluorine per head per day (i.e. 100 p.p.m. F in the diet) for 4 months has been studied.

2. When 100 mg of calcium per head per day were given to the rats, the absorption of fluorine from the alimentary canals has decreased to 55~56% compared with the case of non Ca-added rats.

3. Appearence of mottled teeth in the F-fed rats was recognized after 15 days, but it was done after 30 days in Ca-added rats.

4. The content of fluorine in bones and teeth of F-fed rats has increased to 6~8 times compared with those of normal diet-fed rats, and to 4~5 times in those of F and Ca-fed rats, but there was no effect on the hemoglobin value and on the contents of calcium, magnesium and phosphorus in blood or bones.

5. The hardness and strongness of teeth or bones of F-fed rats have decreased by 15~50% and by 16~29% compared with those of normal rats, respectively. On the other hand, in Ca-added rats they have decreased by only 8~20% and 6~26%, respectively.

Studies on Growth Inhibition of Hiochi-Bacteria, Specific Saprophytes of Saké. Part V. Some Studies on Peptides and Amino Acids as Antagonic Substances of Hydroxyaspergillic Acid. (p. *456~462*)

By Teruo SHIRO and Seiji NAKAMURA
(*Yamamura Saké-Brewing Laboratory*)

In the previous paper[1] the authors isolated hydroxyaspergillic acid from the cultured filtrate of some strains of *Asp. oryzae*, and found it was the majority of the active substances against hiochi-bacteria.

This paper presents a study on peptides and amino acids as antagonic substances to hydroxyaspergillic acid.

The activity of hydroxyaspergillic acid against hiochi-bacteria on Tamura's basal medium[2] is competitively inhibited by the addition of Japanese Saké or polypeptone. In them, peptides and amino acids are naturally contained as growth factors of hiochi-bacteria.

Therefore, the effect of addition of several amino acids on Tamura's medium was examined. When polypeptone was supplemented further to basal medium, *l*-tryptophan and *dl*-valine stimulated the growth of hiochi-bacteria, and *l*-leucine and *dl*-isoleucine inhibited the activity of hydroxyaspergillic acid. When the basal medium was supplemented by Saké, *l*-tryptophan remarkably stimulated the growth of hiochi-bacteria. And *l*-lysine, leucine, isoleucine, threonine and valine inhibited the activity of hydroxyaspergillic acid.

It has been found that the factors inhibit the activity of hydroxyaspergillic acid in Saké are not quite coincident with growth factors of hiochi-bacteria in Saké or polypeptone from view points of heat stability in various pH, dialysis by water, and adsorption in active carbon and ion exchange resins.

1) S. Nakamura and T. Shiro, This Bulletin, **23**, 418 (1959).
2) T. Tamura and Y. Suzuki, *J. Agr. Chem. Soc. Japan*, **32**, 778 (1958).

Studies on the Chemical Combination between Lignin and Carbohydrate. Part VII. Constitution of Lignin-Carbohydrate Complex and Determination Method of Oxidation Products with Nitrobenzene. (p. *462~465*)

By Akira HAYASHI and Isamu TACHI
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Kyoto*)

In order to study the constitution of lignin-carbohydrate complex, acetic acid lignin, acetylated Xylo-lignin, DMF extractive and 50% acetic acid extractive from wheat straw were analyzed.

It was found that the ratio among sugar constituents of these lignin-carbohydrate complex was considerably varied with samples and that the main components of carbohydrate fraction were galactose, glucose, arabinose and xylose. Among these four sugars, xylose was the richest except DMF extractive in which glucose was the richest.

Although the constitution of lignin building stones was not so varied as sugar constitution, *p*-hydroxybenzaldehyde content of lignin-carbohydrate complex was rather higher than that of straw protolignin.

The new method for determining of oxidation product of lignin, consisted of paperchromatography and densitometric estimation after spraying of PAS reagent, was studied and applied for studying of lignin building stone.

Studies on the Chemical Combination between Lignin and Carbohydrate. Part VIII. Successive Extraction of Lignin-Carbohydrate Complex from Straw. (p. *466～468*)

By Akira HAYASHI and Isamu TACHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Kyoto*)

Wheat straw meal was successively extracted with the combination of extracting methods for lignin-carbohydrate complex, resulting four dimethylformamide extractives, 50% acetic acid extractive, and two acetylated Xylolignins.

These samples of lignin-carbohydrate complex extracted successively were analyzed and compared with the corresponding lignin-carbohydrate complex extracted directly from wheat straw.

There was no evident difference between the constitution of successive extractives and that of corresponding direct extractives but the difference owing to isolating method still remained.

It may be suggested that this difference was resulted from the different sort of lignin-carbohydrate complex in wheat straw, and the decomposition such as hydrolysis during isolation did not seem to be the reason of the difference of extractives.

Oxidation of Progesterone and Reichstein's Compound S by Genus *Pseudomonas* and Others. Part II. Studies on Steroid and Microorganisms. (p. *469～472*)

By Hiroshi IIZUKA and Atsushi NAITO

(*The Institute of Applied Microbiology, University of Tokyo*)

It was considered that there were some possibilites in using bacteria, among various microorganisms, for microbiological oxidation of steroids. Optimal conditions of culture were examined prior to oxidation of steroids. The most important point in this reaction was that the addition of a substrate (methanol solution) and inoculation of bacteria to the medium be made at the same time.

Oxidation ability was tested on progesterone and Reichstein's compound S with 162 strains of *Pseudomonas* sp. and 24 strains of *Micrococcus* sp. preserved in this laboratory.

Previously, aproposal was made to classify the genus *Pseudomonas* into three large groups, the fluorescent type, achromonogenic type, and chromogenic type *Pseudomonas*. Achromogenic-type *Pseudomonas* had stronger ability to oxidize steroids than other types. Therefore, this classification of *Pseudomonas* genus seemed to be appropriate even from the characteristics of various strains on oxidation ability of steroids.

(This paper was read at the General Meeting of the Agricultural Chemical Society of Japan, held in Tokyo, in April, 1959)

Production of 6β, 14α-Dihydroxyprogesterone and 6β-Hydroxycomp. S by Bacteria. (Studies on Steroid and Microorganisms. Part III.) (p. *472～475*)

By Hiroshi IIZUKA, Atsushi NAITO, Eiji OHKI, Yoshihiro SATO and Masako HATTORI

(*The Institute of Applied Microbiology, University of Tokyo*)

Oxygenation ability of progesterone and Reichstein's comp. S were compared with various bacteria preserved in our laboratory.

As the results of this experiment, one of these strains (*Achromobacter* sp. strain K 40-5) had stronger oxygenation ability not only progesterone but comp. S.

After examining of culture conditions, mass culture were performed under the best condition.

This oxidation products were 6β, 14α-dihydroxyprogesterone [m.p.=245°, $[\alpha]_D$+125°(c=1.0, $CHCl_3$)], and 6β-Hydroxycomp. S [m.p.=224～226°, $[\alpha]_D$+60.5° (c=0.63, EtOH)] and were gained by bacteria for the first time.

This was read at the General Meeting to the Agricultural Chemical Society of Japan held in Tokyo, in April, 1959.

Studies on Bacterial Amylase. Part XXVIII. Formation of Bacterial Amylase. XI. Influence of Metal Salts on Enzyme Formation. (I) (p. *475～479*)

By Juichiro FUKUMOTO, Takehiko YAMAMOTO, Daisuke TSURU and Mitsugu KAKUMAE

(*Faculty of Science, Osaka City University*)

The effect of metal salts on amylase formation by washed cells of *Bac. amyloliquefaciens* Fukumoto was investigated. It was found that the enzyme formation was promoted by the addition of salts of calcium, strontium or magnesium (10^{-3}M), but markedly inhibited by the addition of zinc or cobalteus salts (1.5×10^{-4}M). The inhibition of enzyme formation induced by zinc salts was reversed by the addition of calcium or strontium salts in amounts of six times that of the former

salts in molar basis. Washing the bacterial cells with a dilute solution of ethylenediamine tetraacetate (M/100 EDTA) lowered calcium content of the cells, simultaneously resulting in a decrease in the amylase-forming ability. However, this decrease was completely recovered by the subsequent addition of calcium or strontium salts. The experiments carried out using $Ca^{45}Cl_2$ revealed that the calcium, which was loosely bounded to the bacterial cells and thus easily removed by EDTA, greatly affected the amount of amylase produced. The amylase produced in the media which contained strontium instead of calcium was purified and obtained in a crystalline state, containing both strontium and calcium. This crystalline amylase was found to differ in some enzymic properties from the bacterial amylase obtained from ordinary culture media.

Antibiotics as Contamination Control Agents in Sake Brewing. Part II. The Composition of Sake Brewed by Using Antibiotics. (p. *479~483*)

By Hiroshi AKIYAMA, Kanichi UMETSU and Mitsuyoshi SHIMIZU

(*Brewing Experiment Station*)

The conventional method of sake brewing belongs to the open fermentation system, and bacterial contamination is controlled by acidification of mash by addition of lactic acid and vigorous yeast fermentation. The authors brewed sake by using antibiotics instead of acidification. The antibiotics used were penicillin and aureomycin. Penicillin was easily destroyed during koji making process and fermentation, however it was effective in controlling bacterial contamination[1].

The composition of the sake obtained was compared to 3 species of commercial sake.

1. The composition of amino acids analyzed by bioassay was no different each other, and the same results by Tamura et al.[2] were obtained.

2. Organic acids were analyzed by silica gel chromatography. There was no difference in succinic acid content, however lactic acid content was very different and by using antibiotics or special brewing method its content was very low.

3. On the contents of various saccharides there were no difference in every sake brewed by different process.

4. From these results it might be possible to mechanize the sake brewing.

1) H. Akiyama, *J. Soc. Brewing Japan*, **53**, 306 (1958).
2) G. Tamura et al., *J. Agr. Chem. Soc. Japan*, **26**, 480 (1952).

Biochemical Studies on the Soil Sickness. Part I. On the Toxic Substance in Fig Roots. (p. *484~486*)

By Yuichi HATSUDA, Sawao MURAO, Noritsugu TERASHIMA and Toshio YOKOTA

(*Department of Agriculture, University of Tottori*)

The soil sickness of fig has long been known. The subject was investigated by Hirai, Hirano and Nishitani since 1949, and it was found that the toxic substance causing soil sickness was contained in stems, leaves and roots of fig plants, and inhibited the seed germination and the seedling growth of radishes.

The authors have carried out experiments on the toxic substance and obtained the following results.

(1) The toxic substance was isolated from the water extract of fig roots.

(2) It proved to have the properties to inhibit remarkably the seed germination and the seedling growth of radishes.

(3) It was confirmed that the substance was identical with psolaren, furo-2′, 3′: 7, 6-coumarin, which was isolated from fig leaves by K. Okahara in 1936.

Biochemical Studies on the Soil Sickness. Part II. On the Toxic Substance in Peach Roots. (p. *486~488*)

By Yuichi HATSUDA, Sawao MURAO, Noritsugu TERASHIMA and Toshio YOKOTA

(*Department of Agriculture, University of Tottori*)

On the soil sickness of peach, Proebsting, Gilmore and Hirano found that the toxic substance causing soil sickness was contained in stems, leaves and roots of peach plants and inhibited the growth of them.

The authors have carried out experiments on the toxic substance and obtained the following results.

(1) The toxic substance was isolated from the water extract of peach roots.

(2) It proved to have the properties to inhibit remarkably the seed germination and the seedling growth of radishes.

(3) It was confirmed that the substance was identical with benzoic acid.

Studies on Taste of Ribonucleic Acid Derivatives. (p. *489~492*)

By Akira KUNINAKA

(*Microbial Laboratory of Yamasa Shoyu Co. Ltd., Chôshi*)

Taste of various ribonucleic acid derivatives was studied. Purine and pyrimidine bases, nucleosides, 2′- and 3′-mononucleotides, and polynucleotides are recognized to have scarce taste. On the other hand, 6-hydroxypurine ribonucleoside-5′-monophosphates (5′-guanylate and 5′-

inosinate) have very agreeable good taste. Both 6-hydroxypurine moiety and ribose-5-phosphate moiety are essential to flavoring action of ribonucleic acid derivatives. Conditions necessary for the structure of nucleotides, which are to be substrates of *Aspergillus* ribosidase, coincide with those necessary for the structure of flavorous nucleotides. The flavorous taste of 6-hydroxypurine ribonucleoside-5′-monophosphates is milder than that of sodium L-glutamate, and is lasted on the gustatory bud much longer than the latter. As additive property is recognized between the taste of 5′-guanylate and that of 5′-inosinate, these nucleotides are considered to have the same kind of taste. There is specific synergy in taste between L-glutamate and 5′-guanylate or 5′-inosinate. This specific synergy is to be basis of application of 6-hydroxypurine ribonucleoside-5′-monophosphates as new seasonings, because most of Japanese foods and beverages contain a rather large amount of L-glutamate.

Studies on Photodecomposition of Amino Acids. (p. *493*～*498*)

By Haruki HARA

(*Food Research Institute, Ministry of Agriculture and Forestry*)

It was found that the following amino acids, such as methionine, tryptophan, histidine, tyrosine, cystine and cysteine, decomposed rapidly under exposure to sunlight with existence of luminescent substances as lumichrome or uranium acetate. These acids also decomposed when casein was subjected to the same treatment. The main substances formed by the photodecomposition were identified as follows: methionine sulfone from methionine, dihydroxy phenylalanine from tyrosine, alanine and anthranilic acid from tryptophan, cysteic acid from cystine, and aspartic acid and urea from histidine.

Studies on the Components of *Ficus carica* L. Part IV. Influence of Psoralene and Bergaptene on the Germination of Some Vegetable Seeds. (p. *498*～*500*)

By Shun-ichi FUKUSHI

(*Department of Agriculture, University of Tottori*)

Previously, the author has reported on the isolation of psoralene and bergaptene from the root of *Ficus carica* L..

In this paper the effects of those furocoumarins on the germination of some vegetable seeds in dark at 24～26°C have been investigated. The results can be summarized as follows.

(1) Psoralene inhibit by 50% the root growth of radish seeds at the dilution of $2/10^{-5}$.

(2) Bergaptene inhibit by 30% the root growth of mustard seeds at the dilution of $1/10^{-5}$.

(3) Psoralene, bergaptene and coumarine are equally active on the sprout growth.

Studies on Silkworm Deoxyribonuclease I. Part II. Mononucleotides Fraction in the Digestion Products of Spleen Deoxyribonucleate. (p. *501*～*506*)

By Jun-ichiro MUKAI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

The mode of the action of silkworm deoxyribonuclease has been investigated. Spleen deoxyribonucleic acid was digested by the purified enzyme at pH 11 in the presence of magnesium ion. The digestion products, after deproteination by shaking with chloroform-octanol mixture and centrifugation, were fractionated by Dowex-1 acetate column chromatography to obtain mononucleotides fraction with 1.3% yield. Free bases or nucleosides appeared in insignificant amount. The eluates containing mononucleotides were combined, desalted and concentrated by charcoal treatment and then chromatographed on filter paper with ammoniacal 70% isopropanol as a developing solvent. Deoxyguanylic acid was thus isolated and determined to be 5′-phosphate as it was dephosphorylated by a snake venom. The rests, having very close R_F values, were refractionated on the resin under a different condition. Deoxycytidylic and deoxyadenylic acids were eluted separately in this way, and each also was identified to be 5′-phosphate by absorption spectrum measurement and venom test. Thymidylic acid could not be detected. These findings were interpreted to indicate that the silkworm deoxyribonuclease hydrolysed, at least as to three mononucleotides isolated, 3′-phosphodiester bonds in the original polynucleotide chain.

Studies on Silkworm Deoxyribonuclease I. Part III. On the Chemical Nature of "Core" Fraction. (p. *507*～*510*)

By Jun-ichiro MUKAI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

The mode of the action of silkworm deoxyribonuclease has been investigated. Spleen deoxyribonucleic acid was digested by the purified enzyme at pH 11 in the presence of magnesium ion. So-called "Core" fraction was prepared by dialysing and then lyophilizing of the digestion product, with a yield of 46%. The following results were obtained about the chemical nature of the fraction:

(1) An average value for the chain length and the

molecular weight is 4.6 and 1450 respectively. The former was determined from the amount of monoesterified phosphate removed by prostatic phosphomonoesterase per total phosphorus, and the latter was calculated from former and the nucleotide composition which was estimated by the usual paperchromatographic method after perchloric acid hydrolysis.

(2) It is a mixture of polynucleotides with monoesterified phosphates at 5′-position. This was verified by the observation that the fraction was nearly completely converted to a mixture of four deoxyribonucleosides in a short incubation with crude venom, and furthermore, the rate of hydrolysis by venom phosphodiesterase as measured by increase in the absorption at 260 mμ of the reaction mixture is remarkably lowered by preincubation with phosphomonoesterase.

Thus the prior postulation was further confirmed that the silkworm nuclease was of a DNase I type that specifically hydrolyses 3′-phosphodiester linkages in the deoxypolynucleotide chain.

On the Fat-Globule Membrane Materials of Cow's Milk. Part V. Interaction between Milk Proteins and Lecithin in Emulsification. (p. *510～513*)

By Susumu KOYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

With a view to studying contribution of milk proteins and lecithin to emulsification of milk, emulsifying power of sodium caseinate, lactalbumin and lactoglobulin in butterfat-phosphate-buffer emulsion was measured under such conditions as (1) only each of these proteins was added, (2) each protein mixed with lecithin was added (3) each protein was added after lecithin was dissolved in butterfat previously.

Results obtained were as follows; Emulsifying power of sodium caseinate was same in (1), (2) and (3), that of lactoglobulin was highest in (3) and lowest in (1), and that of lactalbumin was highest in (1) and lowest in (2). On the other hand, lecithin scarcely showed emulsifying power.

From these results, it was supposed that contribution of lecithin to emulsification of milk was mainly in making more close adsorption of lactoglobulin on the surface of fat globule.

On the Fat-Globule Membrane Materials of Cow's Milk. Part VI. The Properties of Membrane Phosphatase. (p. *514～517*)

By Susumu KOYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

It was previously reported that both fractions (supernatant and precipitate) prepared from the fat-globule membrane materials by means of ultracentrifugation had an alkaline phosphatase activity. In this paper, further investigation of phosphatases contained in both fractions was made in respect of effect of pH on enzyme activity, electrophoretic properties and solubility.

The method of preparation of the fat-globule membrane materials was similar to the technique reported previously. Ultracentrifugation was carried out by means of the Spinco L Type Apparatus.

It was found that there was scarcely difference in the effect of pH on activity between supernatant and precipitate.

After treatment of both fractions with ethanol-ether mixture, the lipid-free proteinous residues were submitted to solubility test and paper electrophoresis. Results obtained showed that the phosphatase contained in supernatant was mostly soluble in 0.08 M NaCl solution but the phosphatase contained in precipitate was mostly insoluble. Furthermore, the former easily migrated on paper electrophoresis, whereas the latter did not migrate.

On the Reaction of Cooked, Stored Meats with 2-Thiobarbituric Acid (TBA). Part I. On the Changes of TBA Values of Cooked Meats during Storage. (p. *518～523*)

By Masao FUJIMAKI and Fujiko YOSHIMATSU*

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

*(*Faculty of Home Economics, Ochanomizu Women's University*)

Cooked, stored meat showed much increased 2-thiobarbituric acid (TBA) values, compared with raw meat or cooked, stored meat added with polyphosphate.

TBA values and peroxide numbers of fats extracted from cooked, stored meats by ether as a solvent showed little change, holding low levels, but TBA values of defatted cooked, stored meats showed much increase during storage of two weeks.

The reacting substances with TBA reagent in cooked, stored meats were little extracted by such an organic solvent as ether, chloroform or ethanol, but most parts of them were found to be extracted by trichloroacetic acid solution.

Fundamental Studies on the Aerobic Fermentation. Part VII. Oxygen Transfer in Gluconic Acid Fermenta-

tion by *Asp. niger*. (p. *523~527*)

By Kôichi YAMADA and Toshimasa YANO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

1. The rate limiting factor in the submerged gluconic acid fermentation by *Asp. niger* van Tieghem strain No. 2 IAM 2093 was found to be the intra-clump resistance against oxygen transfer.

2. The result of the experiments using *Asp. niger* NRRL 337 showed that quotients for oxygen uptake (Q_{O_2}) of mold pellets, diameters of which were larger than 0.3 mm, decreased in hyperbolic manner according to increase of their diameters.

3. On the assumption of homogeneous distribution of mycelium in a pellet, the following conclusion was induced from theoretical consideration that oxygen concentration in a culture medium must be increased in proportion to the square of pellet diameter in order to maintain sufficient oxygen supply up to the interior mycelium of the pellet.

On the Contents of Allantoin- and Uric Acid-Nitrogen in the Urine of Some Mammals.

(p. *528~530*)

By Makoto KANDATSU and Yôko SAITO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The contents of allantoin- and uric acid-N in the urine of rat, rabbit, sheep and goat were determined, for mammals except man and the higher apes excrete allantoin as an end-product of purine metabolism and the new reports about the contents of allantoin in the urine of mammals except rat were unable to find.

Three male adult rats were fed on a stock diet ad lib., one male and one female adult rabbits were fed on a diet containing hay meal and concentrate ad lib., a male adult sheep and a male adult goat were fed on hay and salt ad lib., concentrate 250 g/day.

The urine of the rats, the rabbits, the sheep and the goat contained 6~7 mg/100 g body wt., 3.5~4.5 mg/100 g body wt., 7.5~8.5 mg/kg body wt. of allantoin-N resp., and the urinary N of them contained 3~6% of allantoin-N, but they excreted very little uric acid.

(*continued from front cover*)

Note

Short Communications

Printed by
KASAI PUBLISHING & PRINTING CO
(*Pan-Pacific Press*)
Minato-ku, Tokyo, Japan